

**THE ROLE OF INTEGRINS AND CHEMOKINES IN THE REGULATION  
OF MUCOSAL MAST CELL MIGRATION IN THE MOUSE**

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## **DECLARATION**

This thesis has been composed by myself and the work contained herein is my own.

Anne Rosbottom



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## ABBREVIATIONS

Ab	antibody
Ag	antigen
AMV	avian myoblastosis
AP	alkaline phosphatase
BM	basement membrane
BP	base pairs
BSA	bovine serum albumen
CAPS	3-[cyclohexylamino]-propane-sulphonic acid
CMF HBSS	calcium and magnesium-free hank's balanced salt solution
COPD	chronic obstructive pulmonary disease
CTMC	connective tissue mast cell(s)
DAB	diaminobenzidine
DIG	digoxigenin
DMSO	dimethylsulphoxide
DNP	dinitrophenyl
dNTP	dinucleotriphosphate
dUTP	deoxyuridine-5'-triphosphate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ENA-78	epithelial neutrophil activating peptide-78
FcεRI	high affinity IgE receptor

FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GlyCAM-1	glycosylated cell adhesion molecule-1
hCBMC	human cord blood-derived mast cell
hMC	human mast cell
hPrMC	human mast cell progenitor
HSA	human serum albumen
HUVEC	human vascular endothelial cells
ICAM	intercellular adhesion molecule
IEL	intraepithelial lymphocyte(s)
Ig	immunoglobulin
IL-	interleukin-
IP-10	interferon inducible protein-10
kb	kilobase
kDa	kilodaltons
LBP	laminin binding protein
MAdCAM	mucosal addressin cell adhesion molecule
mBMMC	murine bone marrow-derived mast cell
MCP-1	monocyte chemoattractant protein-1
MIG	monokine induced by IFN- $\gamma$
MIP-1 $\alpha$	macrophage inflammatory protein-1 $\alpha$
MMC	mucosal mast cell(s)
mMCP-1	murine mast cell protease-1



MW	molecular weight
PAF	platelet activating factor
PBS	phosphate buffered saline
PF-4	platelet factor-4
PI3	phosphatidyl inositol-3
PMA	phorbol myristate acetate
PSGL-1	P-selectin glycoprotein ligand-1
RANTES	regulated on activation of normal T-cells, expressed and secreted
RMCP1	rat mast cell protease-I
RT-PCR	reverse transcriptase polymerase chain reaction
SCF	stem cell factor
SDF-1 $\alpha$	stromal cell-derived factor-1 $\alpha$
SDS	sodium dodecyl sulphate
TBS	Tris buffered saline
TECK	thymocyte expressed chemokine
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF- $\beta_1$	transforming growth factor- $\beta_1$
TH	helper T-cell
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumour necrosis factor
VLA	very late after activation

## ABSTRACT

Mucosal mast cells (MMC) play a major role in allergic disease of the gut and in the immune response to gastrointestinal nematodes where, in the mouse, their precursors are recruited into the jejunum and migrate intraepithelially. There, they can be identified as the mature phenotype by their expression of the MMC-specific chymase, mouse mast cell protease-1 (mMCP-1).

Migration of other immune cells is regulated by adhesion to extracellular matrix (ECM) proteins via expression of adhesion molecules such as integrins, and by interactions with chemotactic molecules such as chemokines. Previous work with other mast cell phenotypes suggests mast cells to be no exception to this regulatory system, therefore the aim of this project was to investigate the role of integrins and chemokines in mast cell migration.

Initial experiments analysed, using RT-PCR, expression of potential mast cell chemoattractants from intestinal epithelium following infection of mice with the nematode parasite, *Nippostrongylus brasiliensis*. Chemokines MIP-1 $\alpha$ , RANTES, fractalkine and TECK, and cytokines SCF and TGF- $\beta_1$  were constitutively expressed from intestinal epithelium; and MCP-1 expression was detected only on days 7 and 14 post-infection, coinciding with intraepithelial migration of mast cells. Cultured MMC and the CMT-93 intestinal epithelial cell line expressed some of these molecules, suggesting epithelial cells or intraepithelial MMC as potential sources of mast cell chemoattractants. Furthermore, expression of mRNA for chemokine receptors including CCR1, CCR2, CCR5, CX3CR1 and CXCR4 was detected in

cultured MMC, possibly enabling migration towards chemokines expressed from intestinal epithelium.

Adhesion of cultured MMC to ECM proteins was regulated by TGF- $\beta_1$ , which also regulates the mucosal phenotype, as shown by expression of mMCP-1. MMC cultured with TGF- $\beta_1$  adhered to laminin-1 via expression of the integrin  $\alpha_7\beta_1$ , as demonstrated by RT-PCR, flow cytometry and use of neutralising antibodies. MMC cultured in the absence of TGF- $\beta_1$  adhered to fibronectin and vitronectin but not laminin-1, and did not express  $\alpha_7\beta_1$ . Expression of  $\alpha_7\beta_1$  integrin has not previously been shown in a haemopoietic cell and, as epithelial basement membranes are rich in laminin, this integrin may aid migration and retention of MMC intraepithelially.

In conclusion, this work suggests expression of integrins and chemokine receptors by MMC or their precursors, and of chemokines and cytokines by intestinal epithelium as possible mechanisms regulating intraepithelial migration of MMC.

## CHAPTER 1

### GENERAL INTRODUCTION

Mast cells are specialised granulocytic cells of the immune system, and are the major effector cells of type I hypersensitivity reactions. They were first named "Mastzellen", meaning "well fed cells", by the German medical student, Paul Erlich (Foreman, 1993) because of their prominent granules. The granules were thought to be phagocytosed material, but were later shown to contain the inflammatory mediators central to their role in allergic disease.

#### 1.1 MAST CELLS, ALLERGIC DISEASE AND INFLAMMATORY MEDIATORS

Mast cells play a key role in the pathogenesis of allergic diseases such as asthma, food allergy and ulcerative colitis (Crowe & Purdue, 1992; Page & Minshall, 1993; Miller, 1993). In such diseases, cross-linking of surface-bound IgE by multivalent antigen results in release of a variety of both pre-formed and newly synthesised inflammatory mediators (reviewed in Metcalfe *et al*, 1997). Pre-formed mediators include histamine, proteoglycans such as heparin and chondroitin sulphate, and a number of proteases; those synthesised on mast cell activation include lipid mediators such as prostaglandins and leukotrienes. In addition to these relatively non-specific inflammatory mediators, mast cells also synthesise and release a number of cytokines and chemokines, which may alter the course of the inflammatory response by specific interactions with other immune cells. Studies using human and murine cells have shown expression of cytokines including IL-1 $\beta$ , TNF- $\alpha$ , TGF- $\beta$  and IL-13 (Moller *et al*, 1998; Kanbe *et al*, 1999; Gordon, 2000) and chemokines

including MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1, RANTES and IL-8 (Selvan *et al*, 1994; Lin *et al*, 2000; Oliveira & Lukacs, 2001).

## 1.2 MAST CELLS AND THE IMMUNE RESPONSE TO NEMATODE PARASITES

In addition to their detrimental role in allergic reactions, mast cells are involved in important protective functions including wound healing and, perhaps most importantly, the immune response to gastrointestinal nematodes. Gastrointestinal nematode infection is a major cause of disease world-wide in ruminants, and can also cause disease in humans, in conditions of poor hygiene. In rodents (Miller & Jarret, 1971; MacDonald *et al*, 1980) and man (Miller, 1992), infection with gastrointestinal nematodes results in recruitment and hyperplasia of intestinal mast cells.

Furthermore, mast cells have been shown to be functionally active during nematode infection in the rat (Woodbury *et al*, 1984), and a requirement for mast cells in primary expulsion of the nematode parasite *Trichinella spiralis* has been shown by delayed expulsion in W/W<sup>v</sup> mice, which lack intestinal mast cells (Ha *et al*, 1983).

In some cases however, the mast cell response to nematodes is not protective: although infection of mice with *Nippostrongylus brasiliensis* results in an intestinal mast cell response, W/W<sup>v</sup> mice expel this parasite at the normal rate (Uber *et al*, 1980).

The mast cell response to nematodes includes all the hallmarks of an allergic reaction, including IgE production and release of inflammatory mediators. The role of the majority of these mediators in parasite expulsion is unclear, but mast cell proteases are probably important in nematode parasite expulsion, as demonstrated by

delayed expulsion of *T. spiralis* by mice deficient in the murine mucosal mast cell-specific chymase, mouse mast cell protease-1 (Knight *et al*, 2000). The precise mechanism of action of mast cell proteases in parasite expulsion is unknown, but may involve alterations in epithelial permeability (Scudamore *et al*, 1995). As a type I hypersensitivity response, intestinal nematode infection in rodents provides us with a useful model for study of both immunity to parasites and allergic diseases of the gut.

### 1.3 MAST CELL ORIGIN AND DIFFERENTIATION

Mast cells are distributed throughout the normal connective tissue of organs which interface the environment, such as skin, lung and intestine (Galli, 1990). However, the origin of mast cells and how they arise in these tissues is still a subject of much debate. Unlike basophils, which differentiate in bone marrow and circulate as mature cells (Galli, 1990), no mature mast cells are found in the blood. Kitamura *et al* (Kitamura *et al*, 1981) showed that, in the mouse, mast cells develop from circulating bone marrow-derived spleen colony-forming unit (CFU-S) precursors, which can also give rise to granulocytes. A committed murine mast cell precursor has since been identified as a Thy-1<sup>lo</sup> c-kit<sup>hi</sup> cell which contains cytoplasmic granules and expresses mRNA for mast cell proteases (Rodewald *et al*, 1996). Similarly, the human mast cell precursor was first identified as a CD34<sup>+</sup> progenitor cell (Kirshenbaum, 1991), which could give rise to both mast cells and basophils, but has since been further characterised as a CD34<sup>+</sup>/c-kit<sup>+</sup>/CD13<sup>+</sup> progenitor, from which both mast cells and monocytes arise (Kirshenbaum *et al*, 1999). The factors governing the differentiation of mast cell progenitors into mature mast cells have been extensively studied, and are summarised below.

### 1.3.1 Interleukins and mast cell differentiation

Mast cell dependence on T cell-derived factors was first suggested by *in vivo* experiments using congenitally athymic mice (Ruitenberg & Elgersma, 1976), thymectomised rats (Mayrhofer & Fisher, 1979), and by adoptive transfer of primed T-cells followed by nematode challenge in rats (Nawa & Miller, 1979). Also, several workers have cultured mast cells from haematopoietic cells in media derived from con A-activated T-cells or WEHI-3B murine leukaemia cells (Tertian *et al*, 1981; Yung *et al*, 1981). The major T cell-derived mast cell growth factor has been identified as IL-3 by *in vitro* studies in mice (Razin *et al*, 1984), rats (Haig *et al*, 1988) and humans (Kirshenbaum *et al*, 1992). Evidence of a role for IL-3 in mast cell differentiation *in vivo* came from repetitive administration of IL-3 to nude athymic mice, which resulted in a large increase in intestinal mucosal mast cells (Abe *et al*, 1988). Furthermore, administration of IL-3 induced a mast cell response to the nematode parasite *Strongyloides ratti* (Abe & Nawa, 1988).

Other T cell-derived factors including IL-4 (Hamaguchi *et al*, 1987), IL-9 (Hultner *et al*, 1990; Thompson-Snipes *et al*, 1991) and IL-10 (Thompson-Snipes *et al*, 1991) synergise with IL-3 in promoting mast cell growth, but have no growth promoting properties alone. Notably, the cytokines IL-4, IL-9 and IL-10 are those produced by cells of the T helper 2 (TH2) subset, correlating well with the observation that parasite infection in mice results in a predominantly TH2 type response (Grencis *et al*, 1991; Else & Grencis, 1991; Else *et al*, 1992).

### 1.3.2 Stem cell factor and mast cell differentiation

Stem cell factor (SCF) is a heavily glycosylated protein encoded for by the steel (Sl) locus. It exists in transmembrane and secreted forms (Anderson *et al*, 1990; Kirshenbaum *et al*, 1992), and interacts with a tyrosine kinase linked receptor, *c-kit*, encoded for by the W-locus. Key experiments using W/W<sup>v</sup> and Sl/Sl<sup>d</sup> mutant mice established a role for the gene products of the Sl and W loci in mast cell differentiation before their identity was discovered. Both of these mice are deficient in mast cells, but transplant of bone marrow cells from normal mice or Sl/Sl<sup>d</sup> mice into W/W<sup>v</sup> mice results in differentiation into tissue mast cells (Kitamura *et al*, 1978), whereas the converse experiment was unsuccessful. This is now known to be because W/W<sup>v</sup> mice lack functional *c-kit*, the mast cell growth factor receptor, but the mast cell deficiency of Sl/Sl<sup>d</sup> mice is due to lack of the mast cell growth factor, SCF.

Support of mast cell growth by co-culture of mast cell progenitors with fibroblasts (Ginsburg & Lagunoff, 1967; Levi-Schaffer *et al*, 1986) suggested a fibroblast-derived factor to be involved in mast cell differentiation. Furthermore, lack of responsiveness of mast cells derived from W/W<sup>v</sup> mutant mice to fibroblasts suggested this growth factor to be a ligand for the *c-kit* receptor. This "kit ligand" was subsequently purified from fibroblast conditioned medium (Nocka *et al*, 1990), and fibroblasts are now well recognised as a major source of SCF *in vivo*.

SCF also induces proliferation of murine (Nocka *et al*, 1990) rat (Kitamura *et al*, 1978; Haig *et al*, 1994) and human (Kirshenbaum *et al*, 1992) mast cells *in vitro*, and



synergises with IL-3 as a mast cell growth factor. Treatment of mice with a monoclonal antibody to *c-kit* or SCF, which resulted in a significantly decreased mast cell hyperplasia in response to *T. spiralis* (Grencis *et al*, 1993) and *N. brasiliensis* (Donaldson *et al*, 1996), showed SCF to be a key growth factor in the mast cell response to intestinal nematode parasites.

### 1.3.3 Other factors involved in mast cell differentiation

Other factors which may play a role in mast cell differentiation include the fibroblast-derived nerve growth factor, which acts synergistically with IL-3 to stimulate growth of mouse bone marrow-derived mast cells (mBMMC) *in vitro* (Matsuda *et al*, 1991). Also, interferon- $\gamma$  (Nafziger *et al*, 1990) and TGF- $\beta_1$  (Broide *et al*, 1989) inhibit development of IL-3-dependent mBMMC, although the latter effect is less marked when TGF- $\beta_1$  is combined with IL-3 in combination with IL-9 and SCF (Miller *et al*, 1999).

## 1.4 MAST CELL HETEROGENEITY

Existence of morphological differences between mast cells in different locations in the rat was first recognised 100 years ago (Galli, 1990). However, it was Enerbäck in the 1960s who truly defined mast cell heterogeneity on the basis of differences in histochemical staining. He described "mucosal" mast cells in the intestinal tract and "connective tissue" mast cells of the skin, peritoneal cavity and other sites, in the rat (reviewed in Enerbäck, 1996). Enerbäck and others have since shown that connective tissue (CTMC) and mucosal type mast cells (MMC) differ in a number of other characteristics, as detailed below.

#### 1.4.1 Histochemical characteristics and proteoglycan content

In the rat, Enerbäck reported that, following sequential staining with alcian blue and safranin, MMC stained blue and CTMC took up safranin and stained red (Enerbäck, 1966b). These staining characteristics reflect differences in proteoglycan content of the two cell types; in the rat, CTMC contain heparin (Yurt *et al*, 1977) whereas MMC contain chondroitin sulphate B (Miller *et al*, 1999). Similarly, CTMC in the mouse contain heparin; mucosal mast cells have not yet been purified but a non-heparin proteoglycan content is assumed from their alcian blue<sup>+</sup> / safranin<sup>-</sup> staining characteristics (Miller *et al*, 1999). In general, similar heterogeneity of granule proteoglycans is seen in human mast cell subsets (Metcalf *et al*, 1980; Eliakim *et al*, 1986), but human lung mucosal mast cells appear to contain both heparin and chondroitin sulphate E (Stevens *et al*, 1988).

#### 1.4.2 Protease content

The first example of protease heterogeneity was found in the rat, where connective tissue mast cells of the skin, tongue and intestinal serosa contain the chymase rat mast cell protease I (RMCP I), whereas mucosal mast cells of the jejunal lamina propria and bronchial epithelium contained RMCP II (Gibson & Miller, 1986). In the mouse, more detailed studies revealed a complex picture, with five mast cell chymases (mouse mast cell protease (mMCP)-1, 2, 3, 4 and 5), two tryptases (mMCP-6 and 7) and one carboxypeptidase being reported (Reynolds *et al*, 1990; Schwartz, 1994). mMCPs-3, 4, 6 and mast cell carboxypeptidase are present in CTMC, and mMCP-1 and 2 are markers of the mucosal phenotype (Schwartz, 1994).

Human mast cells can be classified in terms of protease content as those containing only tryptase (MC<sub>T</sub>) and those containing both tryptase and chymase (MC<sub>TC</sub>) (Irani *et al*, 1986; Schwartz, 1994). Mast cells in the lung and intestinal mucosa are predominantly MC<sub>T</sub>, whereas in skin and intestinal submucosa, mast cells are mainly of the MC<sub>TC</sub> type (Irani *et al*, 1986), however as both sites contain representatives of each phenotype, human mast cells cannot be classified by protease content on the basis of tissue location alone.

#### 1.4.3 Functional characteristics

Mast cells also exhibit heterogeneity in terms of their functional characteristics. This was first demonstrated by Enerbäck (Enerbäck, 1966a), who showed that compound 40/80, a known secretagogue for CTMC, failed to cause degranulation of intestinal mast cells. Functional heterogeneity may be important clinically, as one manifestation is varied responses to anti-allergic drugs: sodium chromoglycate and theophylline inhibit IgE-mediated histamine secretion from peritoneal (CTMC) but not isolated MMC (Pearce *et al*, 1982).

In humans, it is not possible to define mast cell phenotype on the basis of functional differences since mast cells of similar phenotype, as defined by protease content, respond differently to secretagogues depending on their anatomical location (Lawrence *et al*, 1987; Liu *et al*, 1990).

## 1.5 REGULATION OF MAST CELL PHENOTYPES

Studies described previously showed that T cell-derived cytokines, particularly IL-3, regulate mast cell differentiation from bone marrow precursor cells; these cytokines also play a role in control of mast cell phenotype.

*In vivo* studies in athymic mice showed differential dependence of CTMC and mucosal mast cells on T-cells (Ruitenberg & Elgersma, 1976); intestinal mast cell hyperplasia in response to *T. spiralis* was absent in these mice, whereas CTMC numbers were comparable to those in control mice. Injection of IL-3 into athymic mice restored the intestinal mast cell response to *S. ratti*, suggesting dependence of MMC on this cytokine (Abe & Nawa, 1988), and *in vitro* studies have also shown the importance of IL-3 in differentiation of MMC in the rat (Haig *et al*, 1988) and mouse (Nabel *et al*, 1981; Schrader *et al*, 1981).

In contrast, SCF appears to promote development of the connective tissue phenotype. When IL-3-dependent mBMMC were cultured with 3T3 fibroblasts, they acquired characteristics indicative of the connective tissue phenotype, including the ability to stain with safranin and a 50-fold increase in histamine content (Dayton *et al*, 1988). Similarly, culture of human CD34<sup>+</sup> pluripotent progenitors with SCF in the presence of IL-3 induced positive staining for berberine sulphate, which stains heparin-containing but not chondroitin sulphate-containing mast cells (Kirshenbaum *et al*, 1992).

The above distinctions, however, are not absolute. W/W<sup>v</sup> and Sl/Sl<sup>d</sup> mice lack both CTMC and MMC indicating a requirement for SCF in differentiation of both mast cell phenotypes, and administration of IL-3 to W/W<sup>v</sup> mice increased cutaneous mast cell numbers, suggesting its importance in differentiation of CTMC. It is likely that *in vivo*, a combination of mast cell growth factors including SCF, IL-3 and other T cell-derived cytokines control mast cell proliferation.

Recent *in vitro* studies in the mouse suggest a role for the multifunctional cytokine TGF- $\beta_1$  in regulation of the mucosal phenotype; these studies are described in more detail in 1.6.5.

## 1.6 USE OF CULTURED MAST CELLS TO STUDY MAST CELL BIOLOGY

Many aspects of mast cell biology are investigated using mast cells cultured *in vitro* from bone marrow cells, or immortalised mast cell lines. There is some confusion with respect to the mast cell phenotype represented by these cells, and the same cells are often used to model both immature and mature mast cells.

### 1.6.1 IL-3-dependent murine bone marrow-derived mast cells

IL-3-dependent murine bone marrow-derived mast cells (mBMMC) have been used to represent immature mast cells (Sriramarao *et al*, 1996; Steegmaier *et al*, 1997), mature differentiated mast cells (Thompson *et al*, 1990) and mucosal type mast cells (Sredni *et al*, 1983; Meininger *et al*, 1992). Phenotypic analysis of these cells shows that they exhibit some characteristics of mucosal type mast cells, including alcian blue staining (Katz *et al*, 1985) and expression of chondroitin sulphate E

proteoglycan (Razin *et al*, 1984). However, these characteristics are also seen in immature CTMC (Combs *et al*, 1965) and are not reliable indicators of the mucosal phenotype. Other studies report expression of CTMC markers carboxypeptidase A and mMCP-5 and -6 (Gurish *et al*, 1992b), but low and variable expression of the mucosal mast cell-specific chymases, mMCP-1 and -2 (Newlands *et al*, 1991; Gurish *et al*, 1992b) by IL-3-dependent mBMMC. Work showing alteration of integrin expression between weeks 3 and 12 of mBMMC may indicate that the 3 - 4 week-old mBMMC used in many studies are not fully differentiated, therefore these cells may be best interpreted as immature stages of both CTMC and MMC.

In some experiments, (Papadopoulos *et al*, 2000) mBMMC cultured with IL-3 or IL-4 and SCF are used to represent CTMC. These cells acquire several characteristics of CTMC, including presence of heparin-staining granules detected by positive staining with safranin.

### 1.6.2 HMC-1 cells

HMC-1 cells are a mast cell line established from a human mast cell leukaemia (Butterfield *et al*, 1988), and are said to represent several cell types including immature mast cells (Juremalm *et al*, 2000; Inamura *et al*, 2002), immature connective tissue mast cells (Kruger-Krasagakes *et al*, 1996) and mature MC<sub>T</sub> tissue mast cells (Nilsson *et al*, 1998). HMC-1 cells express several known mast cell markers including tryptase, histamine, heparin and chondroitin sulphate but no chymase (Nilsson *et al*, 1994a), therefore they are in many ways similar to MC<sub>T</sub>. However, unlike mature mast cells (Shimizu *et al*, 2002), they express no FcεRI and maintain expression of CD13 (Hamann *et al*, 1994). Therefore, as tryptase is thought

to be expressed early in mast cell differentiation (Kirshenbaum *et al*, 1999), HMC-1 cells may also represent immature mast cells but, as neoplastic cells, HMC-1 cells are likely to differ from normal mast cells found *in vivo*.

### 1.6.3 Human cord blood-derived mast cells

A third and most recently characterised type of cultured mast cells are those derived from human cord blood-derived mast cells (CBMC) or BMMC cultured in the presence of SCF, IL-6 ± IL-10 (Yuan *et al*, 1998). In this system, IL-6 and IL-10 synergise with SCF in their effects on mast cell differentiation and IL-10 also minimises proliferation of granulocytes and monocytes. Ochi *et al* (Ochi *et al*, 1999) characterised human (h) CBMC cultured for 4 weeks in SCF, IL-6 and IL-10 as human progenitor mast cells (hPrMC) based on surface expression of *c-kit* and CD13, low level expression of FcεRIα and immunoreactivity for chymase (80%) and tryptase (50%). They also used 9-week-old cells to represent mature human mast cells (hMC), based on higher expression of *c-kit*, continued expression of FcεRI and CD13, toluidine blue staining and immunoreactivity for both tryptase and chymase. Early chymase expression reported by hPrMC differs from other studies which suggest tryptase as an early mast cell marker, and chymase as increasing later in mast cell differentiation (Kirshenbaum *et al*, 1999; Shimizu *et al*, 2002), but this has been attributed to inclusion of IL-10 in cultures. Continued expression of CD13 in cultured hMC, which is not expressed in mature cells (Hamann *et al*, 1994), is also inconsistent with their proposed mature phenotype. 4 week-old hCBMC cultured as described above have similarly been used to represent hPrMC (Boyce *et al*, 2002), and 6 week old cells have been used as a model for mature mast cells (Juremalm *et al*, 2000).

#### 1.6.4 Murine mast cell lines

Numerous murine mast cell lines including PT-18, MC/9, CFTL-15 and CMC<sup>3</sup> have also been used to study mast cell biology and are generally thought to resemble immature mast cells (Galli *et al*, 1982).

Information gained using *in vitro* cultured mast cells should be interpreted with care. The phenotype of these cells may be unclear and their relevance to each individual study should be carefully considered. *In vitro* cultured mast cells are utilised extensively in the studies described below, to investigate mechanisms regulating mucosal mast migration into jejunal mucosa.

#### 1.6.5 mBMMC cultured with IL-3, IL-9, SCF and TGF- $\beta_1$

As detailed above, mBMMC cultured in the presence of IL-3 were initially considered to represent MMC (Sredni *et al*, 1983), but it has been subsequently shown that these cells lack the granule structure seen *in vivo* (Galli, 1990; Metcalfe *et al*, 1997), and express little or none of the MMC-specific chymases, mMCP-1 or -2 (Newlands *et al*, 1991; Gurish *et al*, 1992b). In contrast, murine bone marrow cells cultured with T-cell-conditioned medium (Newlands *et al*, 1991) or in a combination of SCF/IL-9 or SCF/IL-10 (Eklund *et al*, 1993; Xia *et al*, 1996) expressed mMCP-1. Therefore, experiments were set up in our laboratory to investigate the effects of cytokines on differentiation of murine bone marrow cells into cells resembling MMC (Miller *et al*, 1999). These studies showed that culture of mBMMC with WEHI-conditioned medium as a source of IL-3, and with SCF and IL-9 for 7 days resulted in 95% mast cells, of which 20% were positive for expression of mMCP-1 by immunohistochemistry. However, when TGF- $\beta_1$  was added to these cultures, the



percentage of cells expressing mMCP-1 increased to >95% within 4 days. Cells cultured in the presence of TGF- $\beta_1$  also resembled MMC morphologically, in that they were slightly smaller in size and possessed large, electron-dense granules. As TGF- $\beta_1$  is expressed by enterocytes (Barnard *et al*, 1993), T-cells (Letterio & Roberts, 1998) and other cells in the intestinal microenvironment, it was considered that this cytokine could synergise with IL-3, IL-9 and SCF to regulate the mucosal phenotype *in vivo*. Bone marrow mast cells cultured in this way bear the closest resemblance to MMC *in vivo* of any cultured mast cells, and were therefore utilised in the experiments described in chapters 2 - 6 to study potential homing mechanisms of MMC.

## 1.7 MIGRATION OF MUCOSAL MAST CELLS INTO THE JEJUNUM

Mast cells circulate as morphologically unidentifiable precursor cells (Kitamura *et al*, 1981; Kirshenbaum, 1991), but following infection with gastrointestinal parasites such as *N. brasiliensis* (Miller & Jarret, 1971; MacDonald *et al*, 1980) there is an expansion of the jejunal mucosal mast cell population, with increased numbers being located intraepithelially. This is likely to result from both differentiation and division of mast cells within the intestine (Miller & Jarret, 1971), and increased recruitment of the precursors of mast cells from peripheral blood (Kasugai *et al*, 1995).

As they may represent an attractive target for development of novel anti-inflammatory drugs, the mechanisms governing transendothelial recruitment of other leucocytes are relatively well defined (Springer, 1994), and the initial recruitment of mast cell progenitors is likely to share many of these mechanisms. Once in the

lamina propria, mast cells or their progenitors must migrate across the lamina propria and into the epithelium; this stage may share common mechanisms with intraepithelial migration of  $\gamma\delta$  T cells (Smith & Weis, 1996) since both of these cell types home to the intestinal epithelium. Potential mechanisms governing leucocyte trafficking and their application to mucosal mast cell homing are detailed below.

## 1.8 SELECTINS AND RECRUITMENT OF BLOOD-BORNE CELLS ACROSS VASCULAR ENDOTHELIUM

In order to migrate into tissues, cells must attach to and traverse through vascular endothelium. Selectins are a family of calcium-dependent lectin-type adhesion molecules which are involved in the initial interactions of leucocytes with endothelium (reviewed in Springer, 1994). They mediate tethering and rolling onto vascular endothelium via interactions characterised by rapid association-dissociation constants. There are 3 subclasses of selectins: L-selectin is expressed on most circulating leucocytes, whereas E- and P- selectins are expressed on vascular endothelium in response to inflammatory mediators and cytokines; P-selectin is also expressed on platelets. Selectins recognise sialylated carbohydrate counter-receptors; E- and P- selectins interact with carbohydrate structures closely related to sialyl lewis-X, whereas L- and P- selectins recognise O-linked mucin-like molecules such as GlyCAM-1 (glycosylated cell adhesion molecule-1), CD34 and PSGL-1 (P-selectin glycoprotein ligand-1).

The first evidence of selectin involvement in recruitment of mast cell precursors resulted from both *in vitro* and *in vivo* studies using IL-3-dependent mBMMC. Use of a mouse dorsal skin fold model and E- and P-selectin neutralising antibodies

showed rolling of mBMMC on P-selectin (Sriramarao *et al*, 1996) but not E-selectin, and *in vitro* studies supported this result (Steegmaier *et al*, 1997), and revealed PSGL-1 as the major binding site for P-selectin on mBMMC. More recently (Boyce *et al*, 2002), human cord blood cells cultured for 4 weeks in SCF, IL-6 and IL-10 were used to represent mast cell progenitors. These cells expressed PSGL-1 and, in contrast to the above, rolled on both P-selectin and E-selectin though antibodies to PSGL-1 did not block rolling on E-selectin, suggesting involvement of another ligand.

On the basis of the above it is likely that, as in recruitment of other leucocytes, selectins mediate initial interactions of mast cell precursors with vascular endothelium.

## 1.9 INTRODUCTION TO INTEGRINS

Integrins are the major receptors by which cells attach to extracellular matrix and other cells (reviewed in Hynes, 1992; van der Flier & Sonnenberg, 2001), and are expressed by most cells. They are a family of glycosylated, heterodimeric transmembrane adhesion receptors that consist of non-covalently bound  $\alpha$  and  $\beta$  subunits.  $\alpha$  subunits vary in size between 120 and 180 kDa and  $\beta$  subunits are 90 – 110 kDa. Currently, 18  $\alpha$  and 9  $\beta$  subunits are recognised and known to form 24 different heterodimers, and the combination of  $\alpha$  and  $\beta$  subunits determines the ligand specificity of the integrin.

Regulation of cell adhesion via integrins is complicated by the fact that individual integrins can often bind more than one ligand, and the specificity and affinity of a

given integrin receptor on a given cell is not always constant, but can be regulated by both intracellular and extracellular signals. This "inside-out" signalling has been studied for integrin  $\alpha\text{IIB}\beta 3$  in platelets, and requires the activity of Rho-like GTPases (van der Flier & Sonnenberg, 2001). Integrin binding is dependent on divalent cations, the nature of which can affect the affinity and specificity for ligands.

Cell adhesion to the extracellular matrix does not simply serve the purpose of maintaining a cell in a particular location, adhesion may also activate second messenger pathways leading to modulation of cellular function. For example, adhesion of monocytes to extracellular matrix molecules induces genes encoding inflammatory mediators (Sporn *et al*, 1990), thus cell adhesion to the ECM offers a mechanism whereby cellular function can be adapted to local microenvironmental conditions. The roles of integrins in cell migration are detailed below.

### *1.9.1 Integrins and endothelial arrest*

Whilst selectin-mediated rolling represents the first leucocyte-endothelial interaction, in order to begin diapedesis between endothelial cells, a firmer adhesion must be established. In lymphocytes, this firm adhesion is mediated by integrins including the  $\beta 2$  integrins,  $\alpha 4\beta 1$  and  $\alpha 4\beta 7$  (table 1.1). Integrins also add a further regulatory step to leucocyte homing mechanisms: the  $\beta 2$  integrin  $\alpha\text{L}\beta 2$  interacts with intercellular adhesion molecule-1 (ICAM-1) after induction by inflammatory cytokines, whereas interaction with constitutively expressed ICAM2 may be important for leucocyte trafficking in non-inflamed tissues. Furthermore, integrin

Subunits	Name	Ligand
$\alpha$ L $\beta$ 2	LFA-1, CD11a/CD18	ICAM-1, ICAM-2, ICAM-3
$\alpha$ M $\beta$ 2	Mac-1, CD11b/CD18	ICAM-1, fibrinogen
$\alpha$ X $\beta$ 2	P150, CD11c/CD18	Fibrinogen
$\alpha$ 4 $\beta$ 1	CD49d/CD29	VCAM-1, fibrinogen
$\alpha$ 4 $\beta$ 7	CD49d/CD <sup>-</sup>	MAdCAM-1, VCAM-1, fibronectin

**Table 1.1. Integrins involved in leucocyte-endothelial interactions.**

Adapted from Springer, 1994.

Subunits	Ligands
$\beta$ 1	$\alpha$ 1 Collagens, laminin
	$\alpha$ 2 Collagens, laminin
	$\alpha$ 3 Fibronectin, laminin, collagens
	$\alpha$ 4 Fibronectin
	$\alpha$ 5 Fibronectin
	$\alpha$ 6 Laminin
	$\alpha$ 7 Laminin
	$\alpha$ 8 Fibronectin, vitronectin, tenascin <sup>1</sup>
	$\alpha$ v Vitronectin, fibronectin
$\beta$ 3	$\alpha$ v Vitronectin
	$\alpha$ IIb Fibronectin
$\beta$ 5	$\alpha$ v Vitronectin
$\beta$ 6	$\alpha$ v Fibronectin
$\beta$ 7	$\alpha$ 4 Fibronectin

**Table 1.2. Integrins involved in leucocyte-extracellular matrix interactions.**

Adapted from Hynes, 1992

<sup>1</sup> Schnapp *et al*, 1995.

$\alpha 4\beta 1$  is expressed on skin-homing lymphocytes, whereas gut-homing lymphocytes express  $\alpha 4\beta 7$  (Kunkel & Butcher, 2002).

Integrins are also likely to regulate seeding of mast cell progenitors into tissues.

hCBMC cultured in SCF and IL6 constitutively express ICAM-1, VLA-4 and  $\alpha M\beta 2$  (Inamura *et al*, 2001). Furthermore, *In vitro* studies using human cord blood mast cells cultured in SCF, IL-6 and IL-10 to represent mast cell progenitors showed  $\alpha 4$  integrin-VCAM-1 interactions to be important in attachment to TNF- $\alpha$ -activated HUVEC (Boyce *et al*, 2002); the same study also showed expression of  $\alpha L\beta 2$  and  $\alpha M\beta 2$  by cultured mast cell progenitors.

However, the most definitive demonstration of a role for integrins in recruitment of mast cell progenitors has resulted from investigations of  $\alpha 4\beta 7$ -MAdCAM-1 interactions in the mast cell response to intestinal nematode infection. Artis *et al* (Artis *et al*, 2000) showed delayed recruitment of mast cells following *T. spiralis* infection in  $\beta 7$  integrin-deficient mice, and Issekutz (Issekutz *et al*, 2001) demonstrated that blockade of either  $\alpha 4$  or  $\beta 7$  integrins resulted in inhibition of the intestinal mast cell hyperplasia following *N. brasiliensis* infection of rats. The key experiment showing clearly that  $\alpha 4\beta 7$  integrin was required for seeding of mast cell precursors into the small intestine, was performed by Gurish *et al* (Gurish *et al*, 2001), using limiting dilution analysis to measure mast cell precursors in various tissues of  $\beta 7$  integrin-deficient mice. In this experiment, precursors were completely absent from the small intestine but present in lung, spleen and large intestine.

### 1.9.2 Integrins and cell adhesion to the extracellular matrix

Following nematode infection, mast cells or their precursors are thought to migrate across the intestinal epithelium to reside intraepithelially, and it is now well recognised that cell migration and location within tissues is regulated via adhesion to extracellular matrix (ECM) proteins - fibrils or sheets of protein material which contribute to the structural properties of tissues and also have important regulatory functions (detailed in 1.9.3 and 1.9.4). The VLA (very late after activation) integrins (table 1.2) are the major molecules mediating cell adhesion to the extracellular matrix, and expression of these may regulate mast cell migration through tissues.

The VLA integrins are so-named because of the time at which  $\alpha_1\beta_1$  and  $\alpha_2\beta_2$  appear on lymphocytes (Hemler, 1990). However, since most cells in the body express one or more  $\beta_1$  integrins constitutively, and some of the  $\alpha$  subunits associate with other  $\beta$  subunits, the  $\alpha\beta$  nomenclature now is more widely acceptable. In addition to the  $\beta_1$  integrins, the  $\alpha_v$  subunit combines with  $\beta_3$ ,  $\beta_4$ ,  $\beta_5$  and  $\beta_6$  to form molecules which also regulate adhesion to ECM proteins.

Cell adhesion to ECM proteins may be regulated by extracellular signals, for example, the ligand binding affinity of VLA 5 ( $\alpha_5\beta_1$ ) in mast cells was increased by Ag cross-linking of Fc $\epsilon$ RI through a PI3 kinase-dependent mechanism (Kinashi *et al*, 1999). Phorbol esters such as PMA regulate adhesion to the ECM by phosphorylation of cytoskeletal proteins (Danilov & Juliano, 1989) and are often used for stimulation of maximal cell adhesion to ECM proteins. Several integrin subunits including  $\alpha_3$ ,  $\alpha_6$  (Tamura *et al*, 1991) and  $\alpha_7$  (Ziober *et al*, 1993) have

alternatively spliced cytoplasmic domains; some including  $\alpha 6$  (Belkin & Stepp, 2000) and  $\alpha 7$  (Ziober *et al*, 1993) can also be alternatively spliced in their extracellular domains, which may modulate their adhesion properties (Ziober *et al*, 1997; von der Mark *et al*, 2002).

The distribution of ECM proteins varies according to tissue location, therefore mast cell migration from the lamina propria into the epithelium may be regulated by alterations of integrin expression corresponding to differences in ECM proteins between the lamina propria and basement membrane. The ECM proteins rich in these two locations and their integrin ligands are detailed in 1.9.3 and 1.9.4.

### *1.9.3 Integrins and adhesion to extracellular matrix proteins of basement membranes*

Basement membranes support epithelia throughout the body and are composed of a network of proteins including laminin and type IV collagens. Consideration of integrins which might adhere to the BM is pertinent to study of the migration of MMC, as adhesion of mast cells to basement membrane proteins may aid retention intraepithelially.

Laminin is the major non-collagenous component of basement membranes. It consists of a cross-like structure composed of 3 subunits: 2 light chains of approximately 200 kDa, designated  $\beta$  and  $\gamma$ , and a 400 kDa  $\alpha$  chain (Beaulieu & Vachon, 1994). Eleven distinct laminin chains have been identified in humans and mice, and combinations of these generate at least twelve different laminin variants (Kikkawa *et al*, 2000). Expression of the different isoforms is tissue and location-



specific and integrin binding specificities vary between isoforms (Yao *et al*, 1996), though considerable overlap exists.

Due to its ready availability in significant quantities, laminin-1 isolated from the murine Engelbreth-Holm-Swarm tumour is the most commonly studied isoform of laminin. Several regions important in binding of laminin-1 to integrins and other adhesion molecules have been identified using proteolytic fragments and peptides derived from laminin. Most laminin-binding integrins, including  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$  and  $\alpha 7\beta 1$  appear to bind the E8 fragment at the C-terminal of the  $\alpha$  chain (Belkin & Stepp, 2000), whilst the YIGSR region of the  $\beta 1$  chain is involved in adhesion via the non-integrin laminin binding protein (1.9.5) (Graf *et al*, 1987). Other known binding sites include IKVAV (Nomizu *et al*, 1995) and an RGD region (Grant *et al*, 1989).

Opinion on the relevance of laminin-1 to study of cell adhesion to basement membrane laminin *in vivo* is divided. Whereas some authors state laminin-1 as being a major component of the basement membranes of several tissues including small intestine (Simon-Assmann *et al*, 1994; Nomizu *et al*, 1995; De Arcangelis *et al*, 1996), others suggest laminin-1 is uncommon *in vivo* (Falk *et al*, 1999; Kortessmaa *et al*, 2000). Nevertheless, as the different isoforms are likely to have several integrin binding sites in common, laminin-1 represents a convenient model for study of cell adhesion to laminin. Other commonly studied isoforms of laminin include human placental laminin-2/4 or merosin, in which an A-chain variant is present (Delwel *et al*, 1994).

Integrins mediating cell adhesion to laminin include  $\beta 1$  integrins  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 7\beta 1$  and  $\alpha 9\beta 1$ , three members of the  $\alpha v$  subfamily ( $\alpha \beta 3$ ,  $\alpha v\beta 5$  and  $\alpha v\beta 8$ ) and  $\alpha 6\beta 4$  (Belkin & Stepp, 2000).

Type IV collagen is the major collagenous component of basement membranes (reviewed in Olson, 1993a). Molecules are heterotrimers of two  $\alpha 1$  and one  $\alpha 2$  chain and are associated in a network structure. A major binding site has been identified which interacts specifically with integrins  $\alpha 1\beta 1$  and  $\alpha 2\beta 2$  on cells.

#### *1.9.4 Integrins and adhesion to extracellular matrix proteins found in the lamina propria*

Mast cells must migrate across the lamina propria to the epithelium; extracellular matrix proteins which may be found in the lamina propria include fibronectin, vitronectin and fibrillar collagens. Fibronectins are high molecular weight glycoproteins found in many extracellular matrices and in plasma (reviewed in (Hynes, 1993). Subunits are 235 – 270 kDa plus carbohydrate, and each subunit is made up of repeating modules of 2 types. Alternative splicing produces variations in structure and 2 cell binding sites are recognised: the RGD motif is recognised by integrin  $\alpha 5\beta 1$ , and  $\alpha 4\beta 1$  recognises the EILDV sequence.

Vitronectin is an abundant plasma protein produced by the liver (reviewed in (Mosher, 1993). It is also distributed ubiquitously through loose connective tissue, particularly around vascular endothelium (Reilly & Nash, 1988). Vitronectin is

synthesised as a single chain, which is subjected to post-translational modifications before secretion. Its structure includes the RGD cell adhesion sequence, which is recognised by  $\alpha v\beta 1$ ,  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrins.

The main function of fibrillar collagens, including types I - III, V and XI is provision of mechanical support, but they also support cell attachment and differentiation (reviewed in Olson, 1993b). Each molecule is made up of polypeptide units ( $\alpha$  chains), which form triple helices. These form supramolecular aggregates in tissues containing more than one type of collagen, and the resulting fibrils are arranged in different patterns in different tissues. Cells adhere to collagens via integrins  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$  and  $\alpha 3\beta 1$  (Hynes, 1992).

#### *1.9.5 Laminin binding protein and adhesion to the extracellular matrix*

A non-integrin 67 kDa laminin binding protein (LBP) has been shown to be involved in cell adhesion to laminin (Clement *et al*, 1990). It is a dimer, made up of two 37 kDa precursor proteins encoded by a single gene (Rao *et al*, 1989), and binds the YIGSR sequence of laminin (Graf *et al*, 1987). Surface expression of LBP is translationally regulated, therefore cells expressing high levels of LBP mRNA do not necessarily express correspondingly high levels of LBP on their cell surface (Landowski *et al*, 1995). Post-translational modifications may also occur, with surface LBP existing in equilibrium with ribosomal-associated LBP; this is an important consideration when measuring LBP expression, as surface expression cannot be assumed even by detection of protein by Western blotting (Landowski *et al*, 1995).

LBP regulates adhesion of a number of cell types to laminin, including hepatocytes (Clement *et al*, 1990) and T-lymphocytes, where it is co-expressed and co-localises with  $\alpha 6$  integrin (Canfield & Khakoo, 1999). It has diverse roles *in vivo*: LBP is associated with certain tumours in mice (Makrides *et al*, 1988) and humans (Yow *et al*, 1988), and plays a role in embryo implantation in mice (Zhang *et al*, 2000).

#### 1.9.6 Adhesion of mast cells to extracellular matrix proteins

As mast cell adhesion to ECM proteins is likely to regulate their ability to migrate through tissues and thus perform their physiological and pathological functions, it has been extensively studied using cultured and tissue-derived mast cells from rodents and humans (table 1.3). Much of the work on adhesion of murine mast cells to ECM proteins utilised IL-3-dependent mBMMC, which are said to represent an immature phenotype (Stevens *et al*, 1986). These cells adhere to laminin-1 and -2 (Thompson *et al*, 1989a; Thompson *et al*, 1990; Page & Minshall, 1993; Fehlner-Gardiner *et al*, 1996a), fibronectin (Dastyk *et al*, 1991) and vitronectin (Bianchine *et al*, 1992), and integrins  $\alpha 6\beta 1$  (Fehlner-Gardiner *et al*, 1996a; Vliagoftis & Metcalfe, 1997),  $\alpha 4$  and  $\alpha 5\beta 1$  (Fehlner-Gardiner *et al*, 1996b) and  $\alpha v\beta 3$  (Bianchine *et al*, 1992) respectively have been shown to play a role in this adhesion.

Additionally, antibodies to the non-integrin 67 kDa laminin binding protein (LBP) block adhesion of IL-3-dependent mBMMC to laminin-1 (Thompson *et al*, 1989a), indicating a role for this molecule.

As previously described, integrin-mediated adhesion may be regulated by extracellular signals, and stimulation using PMA, Fc $\epsilon$ RI cross-linking or calcium

ECM protein	Cell type	Source	Adhesion mol.	Ref.
Laminin	mBMMC	Mouse bone marrow	$\alpha 6\beta 1$ LBP	Vliagoftis & Metcalfe, 1997 Fehlner-Gardiner <i>et al</i> , 1996a Thompson <i>et al</i> , 1989a
	PT-18	Mouse cell line	LBP	Thompson <i>et al</i> , 1989a
	CFTL-15	Mouse cell line	$\alpha 6\beta 1$	Fehlner-Gardiner <i>et al</i> , 1996a
	MC/9	Mouse cell line	$\alpha 6\beta 1$	Fehlner-Gardiner <i>et al</i> , 1996a
	C57	Mouse cell line	$\alpha 6\beta 1$	Vliagoftis & Metcalfe, 1997
	HMC-1	Human cell line	? <sup>1</sup>	Kruger-Krasagakes <i>et al</i> , 1996
	Human skin MC	Human dermis	$\alpha 3\beta 1$	Columbo <i>et al</i> , 1995
Fibronectin	mBMMC	See above	$\alpha 5\beta 1, \alpha 4\beta 1$ <sup>2</sup>	Fehlner-Gardiner <i>et al</i> , 1996b
	MCP/5	Mouse cell line	$\alpha 5\beta 1$	Dastych <i>et al</i> , 2001
	MC/9	See above	$\alpha 5\beta 1$	Fehlner-Gardiner <i>et al</i> , 1996b
	CFTL-15	See above	$\alpha 4\beta 1, \alpha 5\beta 1$	Fehlner-Gardiner <i>et al</i> , 1996b
	HMC-1	See above	$\alpha 5\beta 1$	Kruger-Krasagakes <i>et al</i> , 1996
	Human skin MC	See above	$\alpha 3\beta 1, \alpha 4\beta 1, \alpha 5\beta 1$	Columbo <i>et al</i> , 1995
Vitronectin	mBMMC	See above	$\alpha v\beta 3$	Bianchine <i>et al</i> , 1992
	Human skin MC	See above	$\alpha v\beta 3$	Columbo & Bochner, 2001

<sup>1</sup> None of the antibodies investigated in this experiment blocked adhesion of HMC-1 cells to laminin.

<sup>2</sup> Both  $\alpha 4\beta 1$  and  $\alpha 5\beta 1$  are involved in adhesion of mBMMC during weeks 1 - 3 of culture.  $\alpha 5\beta 1$  mediates mBMMC adhesion to fibronectin from week 4 onwards.

**Table 1.3. Summary of adhesion to ECM proteins and integrin expression by mast cells.**

mBMMC - murine bone marrow-derived mast cells.

ionophore was necessary for adhesion of IL-3-dependent mBMMC to laminin-1 (Thompson *et al*, 1990) or fibronectin (Dastyh *et al*, 1991), but cells adhered spontaneously to vitronectin (Bianchine *et al*, 1992). Cytokines also stimulated adhesion of mBMMC to ECM proteins: IL-3 stimulated adhesion to laminin (Thompson *et al*, 1989a), and SCF stimulated adhesion to both laminin (Vliagoftis & Metcalfe, 1997) and fibronectin (Dastyh & Metcalfe, 1994).

A number of murine cultured mast cell lines have also been used for study of mast cell adhesion to ECM proteins (table 1.3). PT-18 (Thompson *et al*, 1989a), C57 (Vliagoftis & Metcalfe, 1997), CFTL-15 (Fehlner-Gardiner *et al*, 1996a; Vliagoftis & Metcalfe, 1997) and MC/9 (Fehlner-Gardiner *et al*, 1996a) mast cells adhere to several laminin isoforms, and both LBP (Thompson *et al*, 1989a) and  $\alpha 6$  integrins (Fehlner-Gardiner *et al*, 1996a; Vliagoftis & Metcalfe, 1997) have been implicated in this process. MC/9 (Fehlner-Gardiner *et al*, 1996b) and MCP/5L (Dastyh & Metcalfe, 1994) mast cells adhere to fibronectin via integrin  $\alpha 5$ , but both  $\alpha 4$  and  $\alpha 5$  regulate adhesion of CFTL-15 cells to fibronectin (Fehlner-Gardiner *et al*, 1996b). Although some cultured mast cell lines (Thompson *et al*, 1989a; Fehlner-Gardiner *et al*, 1996a) exhibited spontaneous adhesion, adhesion of other mast cell lines to ECM proteins could be upregulated as in IL-3-dependent mBMMC. The rat basophilic leukaemia cell line (RBL-2H3) and PMA-stimulated rat peritoneal mast cells adhered to fibronectin and vitronectin and expression of integrins  $\alpha 4\beta 1$ ,  $\alpha 5\beta 1$  and  $\alpha v\beta 3$  was detected by immunofluorescent staining. Additionally, antibodies to  $\alpha 4$ ,  $\alpha 5$  or  $\beta 3$  inhibited adhesion of these cells to fibronectin (Yasuda *et al*, 1995).

Study of human mast cell migration through tissues has focussed largely on connective tissue mast cells, due to their proposed role in allergic skin diseases. Consequently, the human mast cell line HMC-1 which, in some studies, is said to resemble human connective tissue mast cells (Nilsson *et al*, 1994a), has been widely used for investigation of the adhesion of human mast cells to ECM proteins. In one study, (Kruger-Krasagakes *et al*, 1996) these cells adhered to human laminin, fibronectin, vitronectin and collagens type I, III and IV; but another study (Nilsson *et al*, 1994c) showed them to adhere only to fibronectin. These differences are reflected by variations in integrin expression between the two studies: Kruger-Krasagakes *et al* (Kruger-Krasagakes *et al*, 1996) showed expression of integrins  $\beta 1$ ,  $\beta 5$ ,  $\alpha 2-6$ , and  $\alpha v$  by HMC-1 cells, and adhesion to fibronectin and vitronectin was blocked by antibodies against integrins  $\alpha 5\beta 1$  and  $\alpha v\beta 5$ , respectively. In contrast, Nilsson *et al* (Nilsson *et al*, 1994c) found only expression of  $\alpha 4\beta 1$  and  $\alpha 5\beta 1$  by the same cell line; the reason for these discrepancies is unclear.

Adhesion properties of mature human skin mast cells have been investigated following extraction of viable cells from tissues, these cells adhered spontaneously to human laminin, fibronectin (Columbo *et al*, 1995) and vitronectin (Columbo & Bochner, 2001). Expression of integrins  $\alpha 3\beta 1$ ,  $\alpha 4\beta 1$ ,  $\alpha 5\beta 1$  and  $\alpha v\beta 3$  but not  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$  or  $\alpha 6\beta 1$  was found in these cells.  $\alpha 3\beta 1$ ,  $\alpha 4\beta 1$ , and  $\alpha 5\beta 1$  were shown, by use of monoclonal antibodies, to be involved in adhesion to fibronectin;  $\alpha 3\beta 1$  antibodies also blocked adhesion to laminin and antibodies to  $\alpha v\beta 3$  showed a role for this integrin in adhesion to vitronectin. Another study (Sperr *et al*, 1992) found that human mast cells isolated from lung and uterus, and HMC-1 cells, expressed the

same spectrum of integrins, with the exception of the  $\alpha 3$  subunit. These integrins are suspected to be involved in location of mast cells within tissues and the similarities in integrin expression may reflect similarities in the areas of ECM in which mast cells accumulate in many tissues (Metcalf *et al*, 1997).

A recent study investigated integrin expression in human intestinal mast cells (Lorentz *et al*, 2002). These cells expressed a similar profile of integrins to those described above, but also expressed  $\alpha 2$  integrin, and adhered spontaneously to fibronectin and laminin-1. However, culture with SCF enhanced by up to 5-fold, adhesion to fibronectin, laminin and collagens, whereas culture with IL-4 reduced adhesion to ECM proteins. Culture of human foetal liver mast cell progenitors with SCF also upregulated mast cell adhesion to vitronectin via expression of  $\alpha v\beta 3$  (Shimizu *et al*, 1995), therefore SCF appears to regulate adhesion of both murine and human mast cells, perhaps reflecting its central role in mast cell function.

As described in other cell types, mast cell adhesion to ECM can modulate cellular function. Adhesion of PMA-stimulated HMC-1 cells to fibronectin or vitronectin enhanced cytokine gene expression and mediator secretion (Kruger-Krasagakes *et al*, 1999) and adhesion of IL-3-dependent mBMMC to vitronectin resulted in an enhanced proliferative response to IL-3 (Bianchini *et al*, 1992), which may be due to phosphorylation of cellular proteins (Bhattacharyya *et al*, 1999).

#### *1.9.7 Integrins and adhesion to intestinal epithelial cells*

In the mouse, >90% of intestinal mast cells are intraepithelial (Miller *et al*, 1995), and it has been suggested that, as is thought to be the case for intraepithelial T-



lymphocytes, expression of specific integrin chains is required for retention of MMC intraepithelially (Smith & Weis, 1996).

The integrin  $\alpha E\beta 7$ , which binds epithelially expressed E-cadherin (Karecla *et al*, 1995), is expressed in > 90% of intraepithelial lymphocytes (IEL) (Cepek *et al*, 1993), and is regulated by TGF- $\beta_1$  (Kilshaw & Murant, 1990; Parker *et al*, 1992). *In vitro* studies showing higher expression of  $\alpha E\beta 7$  on lymphocytes homed into the epithelium (Shibahara *et al*, 2000) suggested a role for this integrin in intraepithelial migration, and reduced expression of  $\alpha E\beta 7$  has been shown in splenic T- cells of Smad7 transgenic mice (Suzuki *et al*, 2002). These mice express Smad7, an intracellular inhibitor of TGF- $\beta$ , and reduced TGF- $\beta$ -mediated expression of  $\alpha E\beta 7$  resulted in a reduction in IEL in these mice, further supporting a role for  $\alpha E\beta 7$  in intraepithelial location of T cells.

Expression of  $\alpha E\beta 7$  which is regulated by TGF- $\beta_1$  or Fc $\epsilon$ RI-cross-linking has been found in IL-3-dependent mBMMC (Smith *et al*, 1994), and TGF- $\beta_1$ -regulated expression of  $\alpha E$  has been shown to correlate with development of the mucosal phenotype in an *in vitro* model of mast cell differentiation (Wright *et al*, 2002). This strongly supports a role for TGF- $\beta_1$ -regulated expression of  $\alpha E\beta 7$  in intraepithelial location of mucosal mast cells, as mature mMCP-1<sup>+</sup> mast cells predominate intraepithelially (Scudamore *et al*, 1997). Furthermore, TGF- $\beta_1$  regulates expression of the  $\beta 1$  integrins in other cells (Ignatz & Massague, 1987), and may therefore upregulate expression of integrins mediating adhesion of mast cells to epithelial

basement membrane proteins, which could further promote intraepithelial location of MMC.

## 1.10 CHEMOATTRACTANTS AND CELL MIGRATION INTO AND THROUGH TISSUES

Whilst selectins and integrins facilitate adhesion of emigrating leucocytes to vascular endothelium, before they migrate from the circulation into tissues, the cells must receive chemotactic stimuli from molecules expressed by vascular endothelium or by other cells within these tissues. Further leucocyte migration through tissues, including migration of mast cells into intestinal epithelium, is also likely to be regulated by chemotactic molecules.

A number of molecules have been identified which are chemotactic for leucocytes. Classical chemoattractants include bacterial-derived N-formyl peptides, complement fragments C3a and C5a and lipids such as leukotriene B4 and PAF (Murdoch & Finn, 2000). Recently, a family of chemotactic cytokines named "chemokines" have been discovered (Taub & Oppenheim, 1993) and shown to be involved in leucocyte recruitment into tissues.

### *1.10.1 Chemokines and chemokine receptors*

Chemokines are low molecular weight (8 - 17 kDa) proteins which can be divided into 4 groups (CXC, CX3X, CC and C) according to the positioning of two N-terminal cysteine residues (Rottman, 1999; Murdoch & Finn, 2000). Their effects on target cells are mediated by interaction with specific 7-transmembrane spanning G-protein coupled receptors, which are generally classified according to their

chemokine ligands. CXCR1 - 5 bind CXC chemokines, whereas CCR1- 9 bind a number of CC chemokines. Additionally, receptors for fractalkine (CX3CR1) and lymphotactin (XCR1) have been identified.

Chemokines are unique in their promiscuous interactions with their receptors (Mantovani, 1999); one chemokine can bind to various receptors, whilst target cells can express several different chemokine receptors. This feature is thought to render the chemokine system more robust, so that loss of one particular chemokine or receptor due to a gene polymorphism would be less deleterious to the system.

Although much redundancy exists in the system, chemokines do exhibit some specificity in the cell types with which they interact. CXC chemokines generally recruit neutrophils, whereas CC chemokines act primarily on monocytes and T-cells (Conti *et al*, 1999). Furthermore, chemokine receptors are differentially expressed on TH1 and TH2 cells, thus explaining subset-specific T-cell recruitment in diseases characterised by a particular inflammatory response. TH1 cells express chemokine receptors CCR5 and CXCR3 and preferentially accumulate in synovial fluid of patients with the TH1-mediated disease rheumatoid arthritis, whereas expression of CCR3, CCR4 and CCR8 mediates accumulation of TH2 cells in allergic-type diseases such as asthma (Rottman, 1999; Homey & Zlotnik, 1999).

#### *1.10.2 Chemokines and leucocyte recruitment*

Chemokines are a critical part of the multi-step process whereby leucocytes are recruited across the vascular endothelium into tissues. In addition to provision of a chemotactic stimulus, chemokines activate  $\beta 2$  integrins such as CD11b/CD18

expressed by rolling leucocytes, leading to firm adhesion or "arrest" on endothelium via interactions with counterligands such as ICAM-1, -2 and -3 (Springer, 1994; Murdoch & Finn, 2000). It is thought that binding of chemokines to endothelium via heparin binding sites may allow establishment of a concentration gradient, even in flow conditions (Springer, 1994). Arrest chemokines or chemokine receptors have been identified for several leucocytes: monoclonal antibodies to CCR3 blocked eosinophil arrest on endothelium stimulated by inflammatory mediators under shear flow conditions (Kitayama *et al*, 1998). Similarly, several chemokines stimulate lymphocyte arrest under flow conditions: SDF-1, 6-C-kine and MIP-1 $\alpha$  triggered adhesion of memory CD4<sup>+</sup> T-cells (Campbell *et al*, 1998).

Although a specific arrest chemokine for mast cells has not been identified, chemokine-mediated transmigration of mast cells through endothelium has been shown *in vitro* (detailed in 1.11.1), and may regulate accumulation of mast cell precursors into tissues.

### *1.10.3 Chemokine expression and inflammatory disease*

Upregulation of chemokine expression has been shown in a number of inflammatory and allergic-type diseases, and is thought to regulate the specific inflammatory responses seen in these diseases. Some of the chemokines upregulated in these diseases are potential mast cell chemoattractants.

In asthmatic patients, upregulation of eotaxin has been shown in lung epithelial and endothelial cells, and mucosa-infiltrating eosinophils expressing the eotaxin receptor CCR3 have been identified (Homey & Zlotnik, 1999). Expression of CCR3 has also

been demonstrated in allergic dermatitis. Other chemokines associated with the pathogenesis of asthma include RANTES, MCP-1, -3 and -5 and MIP-1 $\alpha$  (Homey & Zlotnik, 1999; Teran, 2000).

Several chemokines are upregulated in inflammatory diseases of the gut. As mast cells may play a role in many of these diseases (Crowe & Purdue, 1992), this suggests a possible role for chemokines in intestinal recruitment of mucosal mast cells. Increased expression of the CC chemokines MCP-1 and RANTES has been shown in inflammatory bowel disease (IBD) using RT-PCR, *in situ*-hybridisation and immunohistochemistry. Expression of MCP-1 was upregulated in venule endothelial cells, spindle cells and mononuclear cells (Mazzucchelli *et al*, 1996), and RANTES expression was upregulated in IEL and the subendothelial lamina propria (Reinecker *et al*, 1995). In ulcerative colitis, increased immunohistochemical staining for MIP-1 $\alpha$  was shown in colonic epithelial cells, macrophages and neutrophils (Vainer *et al*, 1998); and expression of fractalkine mRNA was upregulated in intestinal epithelial and endothelial cells during active Crohn's disease (Muehlhoefer *et al*, 2000). Expression of CC chemokines may contribute towards the chronic inflammation seen in such diseases by recruitment of inflammatory cells such as monocytes and memory T-cells. In contrast, CXC chemokines may contribute to the acute phase of inflammatory bowel diseases. Increased expression of IL-8 has been identified in the mucosa of patients with active Crohn's disease and ulcerative colitis, and this has been correlated with increased numbers of neutrophils (Macdermott, 1999). Increased expression of ENA-78 has also been detected in

epithelial cells of patients with lower grade disease activity, and may contribute further towards attraction of granulocytes into IBD mucosa (Macdermott, 1999).

#### *1.10.4 Chemokines, epithelium and the immune response to gut-dwelling pathogens*

In order to attract mast cells into epithelium, the relevant chemoattractants would be expected to be epithelially-derived. Epithelium is well-established as a source of chemokines, which may be expressed by both epithelial cells and intraepithelial cells such as lymphocytes. Chemokines such as TECK, which are expressed constitutively by intestinal epithelial cells (Wurbel *et al*, 2000), are suspected to be involved in homeostatic lymphocyte trafficking into intestinal mucosa (Kunkel *et al*, 2000; Luster, 2001). However, several epithelial cell-derived chemokines, though constitutively expressed, are upregulated in inflammatory diseases or in response to cytokines, and their expression is thought to attract inflammatory cells into the epithelium and mucosa. Stimulation of freshly-isolated human colon epithelial cells with IL-1 $\alpha$  resulted in upregulation of expression of neutrophil chemoattractants GRO- $\alpha$ , GRO- $\gamma$  and ENA-78 and monocyte/T-cell chemoattractants MCP-1 and RANTES (Yang *et al*, 1997). Furthermore, expression of IP10 and MIG was upregulated in human epithelial cell lines in response to interferon- $\gamma$  and resulted in chemotaxis of intraepithelial lymphocytes (Dwinell *et al*, 2001; Shibahara *et al*, 2001). IEL also migrate towards chemokines IL-8, GRO- $\alpha$ , MIP-1 $\alpha$ , MCP-1 (Roberts *et al*, 1997) and fractalkine (Muehlhoefer *et al*, 2000), all of which may be expressed by intestinal epithelial cells. IEL can also express several chemokines and cytokines themselves, including MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES, IFN- $\gamma$ , TNF- $\alpha$  and

TGF- $\beta_1$  (Boismenu *et al*, 1996; Kearsey & Stadnyk, 1996), and may therefore contribute to epithelially-regulated cellular recruitment.

Expression of chemokines from intestinal epithelium can be upregulated in response to gastrointestinal infections, and intraepithelial migration of mast cells following *N. brasiliensis* could be regulated by parasite-induced expression of the relevant chemokines by epithelium. Interaction of bacteria, viruses and nematode parasites with intestinal epithelium can stimulate upregulation of chemokines and cytokines, both of which may promote influx of inflammatory cells. Infection of human colon epithelial cell lines with invasive bacteria resulted in upregulation of IL-8, MCP-1, GM-CSF and TNF- $\alpha$  (Jung *et al*, 1995), and rotavirus infection of the human epithelial cell line HT-29 stimulated upregulation of CC chemokines MIP-1 $\alpha$ , MCP-1 and RANTES and of CXC chemokines IL-8, IP10 and GRO- $\alpha$  (Rollo *et al*, 1999).

The epithelial response to the nematode parasite *T. spiralis* has been studied using both cell culture (Li *et al*, 1998) and epithelial isolation methods (Stadnyk & Kearsey, 1996), where upregulation of IL-1 $\beta$ , IL-8 and ENA-78 have been shown. These chemokines are likely to initiate the neutrophil response to this parasite. Expression of chemokines and cytokines in response to *N. brasiliensis* has not been studied, but upregulation of mast cell chemoattractants could contribute to mast cell recruitment into the epithelium.

## 1.11 MAST CELL CHEMOATTRACTANTS

As the ability to migrate into and through tissues is likely to be critical to the function of both CTMC and MMC, several molecules have been investigated for mast cell chemoattractant activity based on their chemoattractant activity for other leucocytes. Mast cell types including mBMMC, hCBMC, HMC-1 cells, rat peritoneal mast cells and human tissue mast cells have been found to undergo directed migration towards a range of molecules including both classical chemoattractants such as complement fractions, and a number of chemokines. Mast cell migration is usually quantified using a Boyden chamber technique, where migration through polycarbonate filters is measured. As migration of mast cells is dependent on adhesion to extracellular matrix proteins, filters are coated with laminin (Taub *et al*, 1995; Hartmann *et al*, 1997), fibronectin (Nilsson *et al*, 1994c) or vitronectin (Taub *et al*, 1995).

As reported for other cells (Haribabu *et al*, 1999), interaction of mast cell chemoattractants with their receptors activates G-protein signalling pathways, as shown by use of specific inhibitors such as pertussis toxin, leading to cytoskeletal rearrangements which result in cell migration.

Mast cell influx into tissues has been correlated with tissue expression of chemoattractants, supporting an *in vivo* role in mast cell recruitment. A number of mast cell chemoattractants are detailed below.

### 1.11.1 Chemokines and chemokine receptor expression by mast cells

The importance of chemokines in recruitment of inflammatory cells is now well established, and a growing body of evidence suggests that mast cells, similarly,



respond to chemokines. Expression of a number of chemokine receptors, and chemotaxis towards their chemokine ligands has been found in mast cells (table 1.4). Additionally, correlation of chemokine expression with increased mast cell numbers in disease states points towards a prominent role for chemokines in mast cell recruitment *in vivo*.

The first evidence of mast cell chemoattractant activities of chemokines came from experiments by Taub *et al* using IL-3-dependent mBMMC (Taub *et al*, 1995). These cells migrated towards concentration gradients of MCP-1 and RANTES and, following IgE activation, they also migrated towards PF4 and MIP-1 $\alpha$ . The importance of several of these molecules in mast cell recruitment *in vivo* has also been demonstrated. Intramuscular injection of human recombinant RANTES in mice resulted in an influx of mast cells within 4 h (Conti *et al*, 1998), and expression of RANTES in a rat model of colitis was correlated with increased mast cell numbers (Ajuebor *et al*, 2001). Similarly, injection of MCP-1 into rat skin resulted in recruitment of basophilic granular cells (Conti *et al*, 1997) and expression of MCP-1 in human wound healing (Trautmann *et al*, 2000) and in chronic obstructive pulmonary disease (COPD) (de Boer *et al*, 2000) correlated with increased numbers of mast cells.

More recently, expression of corresponding chemokine receptors to the above chemokines and to other CC chemokine ligands was demonstrated in mBMMC which were cultured with IL-3 and SCF. In this experiment, expression of chemokine receptors CCR1 - 5 were shown by RT-PCR, and all except CCR4 were

Chemokine	Chemotactic for-	Receptor	Approx. conc.	Ref.
IL-8	HMC-1 hCBMC Human skin mast cells <sup>1</sup> hPrMC <sup>1</sup> (CBMC)	CXCR1, CXCR2 " " CXCR2	50 ng/ml	Nilsson <i>et al</i> , 1999 Lippert <i>et al</i> , 1998; Inamura <i>et al</i> , 2002 Ochi <i>et al</i> , 1999
RANTES	mBMMC <sup>2</sup> , Murine peritoneal MC	?	50 ng/ml	Taub <i>et al</i> , 1995
PF-4				
MCP-1	mBMMC <sup>3</sup>	CCR2		Oliveira & Lukacs, 2001
MIP-1 $\alpha$	"	CCR1		Taub <i>et al</i> , 1995
MIP-1 $\beta$	mBMMC <sup>3</sup> , hPrMC <sup>1</sup> (CBMC)	CCR5		As above + Ochi <i>et al</i> , 1999
SDF-1 $\alpha$	HMC-1 hCBMC hPrMC <sup>1</sup> (CBMC)	CXCR4 " "	1 $\mu$ g/ml 100 ng/ml	Juremaln <i>et al</i> , 2000; Lin <i>et al</i> , 2000 Ochi <i>et al</i> , 1999
Eotaxin	human skin mast cells hPrMC (CBMC) hCBMC mBMMC	CCR3 " " "	100 ng/ml 100 nM	Romagnani <i>et al</i> , 1999 Ochi <i>et al</i> , 1999 Oliveira & Lukacs, 2001
Fractalkine	mBMMC <sup>4</sup> Human skin mast cells	CX3CR1 "	25 ng/ml	Papadopoulos <i>et al</i> , 2000

<sup>1</sup> Expression of receptor only.

<sup>2</sup> IL-3-dependent mBMMC were used in this study.

<sup>3</sup> Chemotaxis towards these ligands shown in IL-3-dependent mBMMC, and both receptor expression and chemotaxis towards relevant ligands shown in mBMMC cultured with IL3 and SCF.

<sup>4</sup> mBMMC used in this study were cultured with IL3 or IL4/SCF.

**Table 1.4. Summary of mast cell chemoattractant chemokines, and expression of the corresponding chemokine receptors by mast cells.**

mBMMC - murine bone marrow-derived mast cells, hCBMC - human cord blood-derived mast cells, hPrMC (CBMC) - human progenitor-like mast cells, derived from cord blood. See table 1.3 for source of mast cells.

functional. Furthermore, expression of CCR1, CCR2, CCR3 and CCR5 was upregulated by IgE or SCF (Oliveira & Lukacs, 2001).

Studies of CC-chemokine receptor expression in human mast cells have focussed on the eotaxin receptor, CCR3, as its ligand eotaxin is associated with allergic disease (Homey & Zlotnik, 1999), and CCR3 expression is shared by other cells central to allergic inflammation, such as eosinophils, basophils and TH2 lymphocytes.

Tryptase-chymase double-positive mast cells of skin and intestinal submucosa expressed CCR3, and mast cells isolated from human lung underwent chemotaxis towards eotaxin (Romagnani *et al*, 1999; De Paulis *et al*, 2001). Ochi *et al* (Ochi *et al*, 1999) cultured human cord blood-derived mast cells with SCF, IL-6 and IL-10 to study chemokine receptor expression during mast cell differentiation. They reported that CCR3 was the only chemokine receptor expressed by cells from 9 week-old cultures, which resembled mature hMC, but in 4 week-old cells resembling mast cell progenitors, expression of CXCR2, CXCR4, CCR3 and CCR5 was detected by RT-PCR and flow cytometry.

In contrast to the above, Juremalm *et al* (Juremalm *et al*, 2000) reported expression of CXCR4 and migration towards its ligand SDF-1 $\alpha$  in both HMC-1 cells, which were used to represent immature mast cells, and CBMC cultured for 6 weeks in SCF and IL-6, and said to represent a more mature mast cell. This discrepancy was explained by the authors as possibly being due to different culture protocols; IL-10 was not included in this study. Another study (Lin *et al*, 2000) also reported expression of CXCR4 in CBMC cultured with SCF and IL-6 (for 8 weeks), and

showed transmigration of these cells through human umbilical vein endothelial monolayers in response to SDF-1 $\alpha$ , thus demonstrating a possible functional role for SDF-1 $\alpha$  in seeding of mast cells into tissues.

Expression of the CXC receptors CXCR1 and CXCR2, which in humans are the receptors for IL-8, has been reported in cultured human mast cells. One study (Nilsson *et al*, 1999) reported HMC-1 cells to express CXCR2 only and migrate towards IL-8, whereas an earlier study (Lippert *et al*, 1998) reported expression of both functional CXCR1 and CXCR2 by HMC-1 cells. A recent study showed expression of CXCR1 and CXCR2 in hCBMC cultured with SCF and IL-6, using RT-PCR and flow cytometry; these cells also underwent chemotaxis towards IL-8 (Inamura *et al*, 2002).

The CX3C chemokine fractalkine is also chemotactic for "mucosal" and "connective tissue" mast cells cultured from murine bone marrow cells in IL-3, and IL-4 and SCF, respectively (Papadopoulos *et al*, 2000). Both cell types expressed mRNA for the fractalkine receptor CX3CR1, as did mast cells isolated from human skin.

#### *1.11.2 Cytokines as mast cell chemoattractants*

A number of the cytokines which play a role in mast cell growth and differentiation are also chemotactic for mast cells (table 1.5). SCF is chemotactic for HMC-1 cells, human SCF-dependent CBMC (Nilsson *et al*, 1994b), murine mast cells cultured in spleen cell-conditioned medium and murine peritoneal mast cells (Meininger *et al*, 1992). Also, expression of SCF has been correlated with mast cell accumulation in

human arthritic synovial fluid (Ceponis *et al*, 1998) and in nasal lavage fluid in pollen allergic rhinitis (Nilsson *et al*, 1998).

The chemotactic activity of TGF- $\beta_1$  for mast cells further supports a key role in regulation of mast cell migration into epithelium. Studies using murine peritoneal mast cells and the CMC<sup>3</sup> mast cell line showed TGF- $\beta_1$  to be an extremely potent mast cell chemoattractant, active at femtomolar concentrations (Gruber *et al*, 1994). IL-3 is the key T-cell-derived mast cell growth factor and also exhibits chemotactic activity towards murine peritoneal mast cells (Matsuura & Zetter, 1989).

#### *1.11.3 Classical chemoattractants and mast cell chemotaxis*

Classic neutrophil chemoattractants including complement fractions C3a and C5a, and platelet activating factor (PAF) are also chemotactic for mast cells (table 1.5). Investigations using HMC-1 cells showed both C3a and C5a to be active at picomolar concentrations via distinct G protein-coupled receptors (Nilsson *et al*, 1996). Additionally, human CBMC cultured in SCF and IL-6 and freshly isolated human skin mast cells were chemoattracted by complement fractions (Hartmann *et al*, 1997).

More recently, PAF has also been shown to be a chemoattractant for HMC-1 cells, active at nanomolar concentrations (Nilsson *et al*, 2000).

#### *1.11.4 Other mast cell chemoattractants*

Antibacterial peptides induce mast cell chemotaxis; both LL-37 and  $\beta$ -defensin-2 stimulated degranulation and chemotaxis of rat peritoneal mast cells

Molecule	Chemotactic for-	Approx. conc.	Ref.
Complement C3a, C5a	HMC-1 mast cell line hCBMC Skin MC	Picomolar	Nilsson <i>et al</i> , 1996 Hartmann <i>et al</i> , 1997
C1q	C57 HMC-1	Biphasic 0.1 nM, 50 nM	Ghebrehiwet <i>et al</i> , 1995
PAF	HMC-1 hCBMC mBMMC	nM	Nilsson <i>et al</i> , 2000
SCF	HMC-1	50 ng/ml	Nilsson <i>et al</i> , 1994b
	hCBMC		
	hBMMC	1 ng/ml	Meininger <i>et al</i> , 1992
	Murine peritoneal MC		
TGF- $\beta_1$	Murine peritoneal MC CMC <sup>3</sup> mast cell line	fM	Gruber <i>et al</i> , 1994
IL-3	Murine peritoneal MC	100 U/ml	Matsuura & Zetter, 1989
Angiogenic factors	C57	pM	Gruber <i>et al</i> , 1995
sAA	HMC-1 hCBMC	$\mu$ M	Olsson <i>et al</i> , 1999
Adenine nucleotides	Rat peritoneal MC	$\mu$ M	McLoskey <i>et al</i> , 1999
Laminin	mPT-18 mBMMC	250 $\mu$ g/ml	Thompson <i>et al</i> , 1989b
Specific Ag	RBL	100 ng	Orida <i>et al</i> , 1983
LL-37	Rat peritoneal MC	5 $\mu$ g/ml	Niyonsaba <i>et al</i> , 2002b
$\beta$ -defensin-2	Rat peritoneal MC	3 $\mu$ g/ml	Niyonsaba <i>et al</i> , 2002a

**Table 1.5. Summary of mast cell chemoattractants.**

mBMMC - murine bone marrow-derived mast cells, hCBMC - human cord blood-derived mast cells, RBL - rat basophilic leukaemia cell line. See table 1.3 for the sources of mast cells.

(Niyonsaba *et al*, 2002a; Niyonsaba *et al*, 2002b). These peptides are widely expressed in epithelial tissues, where mast cells may be present, and are upregulated in inflammatory diseases and so may contribute towards mast cell accumulation. Mast cells also undergo chemotaxis towards laminin, a major component of basement membranes. Murine PT18 mast cells are attracted towards laminin and the synthetic peptide PA22-2, which corresponds to the active site on the A-chain of laminin (Thompson *et al*, 1989b). Other mast cell chemoattractants include angiogenic factors (Gruber *et al*, 1995), adenine nucleotides (McLoskey *et al*, 1999), serum amyloid A (Olsson *et al*, 1999) and specific antigen (Orida *et al*, 1983). The list continues to grow as more potential mast cell chemoattractants are investigated.

#### 1.12 AIMS OF THIS STUDY

Whilst considerable advances have been made in the field of mast cell trafficking, important questions remain unanswered. Previous work on mast cell trafficking into the intestinal mucosa has focussed on seeding of mast cell precursors across vascular endothelium into tissues (Artis *et al*, 2000; Issekutz *et al*, 2001; Gurish *et al*, 2001). Mechanisms whereby mast cells or their precursors migrate through the intestinal lamina propria, but particularly into intestinal epithelium, are poorly understood.

Upregulation of expression of mast cell chemoattractants from intestinal epithelium may regulate the mast cell response to gastrointestinal nematode infection. Epithelial expression of neutrophil chemoattractants has been shown in response to *T. spiralis* (Stadnyk & Kearsey, 1996; Li *et al*, 1998) but epithelial expression of mast cell chemoattractants following gastrointestinal nematode infection has not been investigated.

Expression of chemokine receptors which may play a role in murine mast cell migration has also been investigated in cultured mBMMC but, as described in 1.6.1, these cells are likely to resemble immature mast cells, therefore their relevance to intraepithelial migration of MMC is questionable. Similarly, previous studies of integrin expression in mast cells may have revealed molecules likely to regulate migration through connective tissues of the lamina propria. However, study of the integrins controlling intraepithelial migration or retention of mature mucosal mast cells intraepithelially is likely to be made more relevant by use of a cultured MMC homologue (Miller *et al*, 1999).

The aims of this study therefore, were to characterise mechanisms controlling intraepithelial migration of murine MMC, utilising mBMMC cultured as described (Miller *et al*, 1999) (1.6.5) to represent MMC where appropriate. In order to do this, three hypotheses have been formed and tested.

The first hypothesis was that gastrointestinal nematode infection would result in upregulation in expression of mast cell chemoattractants by intestinal epithelium. Secondly, it was hypothesised that mucosal type mast cells would express the relevant receptors to respond to such mast cell chemoattractants. The final hypothesis was that, in regulation of the mucosal phenotype, TGF- $\beta_1$  might also regulate the expression of integrins which might support intraepithelial migration of mucosal mast cells



## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 ANIMALS

12-week-old male BALB/c mice were obtained from Bantam and Kingman Universal, Hull, U.K. and maintained at the Centre for Tropical Veterinary Medicine animal house, University of Edinburgh, on *ad libitum* water and standard pelleted food.

#### 2.2 PARASITOLOGICAL TECHNIQUES

##### 2.2.1 Infection of mice with *Nippostrongylus brasiliensis*

The mouse-adapted strain of *Nippostrongylus brasiliensis* was maintained as described previously (Nawa & Miller, 1978). Briefly, 7 days after infection faeces were collected into deionised water, washed and mixed to form a thick paste. A pre-autoclaved 50:50 mix of washed granular charcoal (10-18 mesh, BDH, Poole, Dorset, U.K.) and peat moss was added to the faeces and the mixture incubated at 26°C in paper-lined petri dishes placed in a humidified plastic box. After 7 - 28 days of incubation, the L<sub>3</sub> larvae were allowed to migrate for 5 min into warm saline in the petri dish. They were then removed into a larger volume of warm saline and collected using the Baermann technique, with live larvae migrating through 2 layers of laboratory tissue held in a filter funnel into the clamped end of a drainage tube. Suspensions of larvae were then counted under a dissecting microscope, and the volume adjusted so that 500L<sub>3</sub> larvae in a volume of 0.2 ml could be injected subcutaneously into the flank of mice under halothane anaesthesia.

### 2.2.2 *Faecal egg counts*

When required to confirm infection, faeces were collected overnight on day 6 of infection. 1 g of faeces was mixed with 15 ml of saturated salt solution and the eggs separated using a tea strainer and counted in a Mc Master chamber.

## 2.3 COLLECTION AND PREPARATION OF SAMPLES FROM *NIPPOSTRONGYLUS BRASILIENSIS* INFECTED MICE

### 2.3.1 *Serum samples*

Serum was obtained by collecting blood from the brachial artery immediately after death but prior to perfusion (2.3.2). Blood was allowed to clot for 1 h at 37°C, then stored overnight at 4°C to aid clot retraction. Serum was then aspirated into fresh tubes, centrifuged at 13000g for 5 min, and the supernatant stored at -70°C before use.

### 2.3.2 *Collection of murine jejunal epithelium*

Epithelium was stripped from a section of proximal jejunum using a modification of the method previously described by Bjerknes *et al* (Bjerknes & Cheng, 1981). A solution of 24 mM ethylenediaminetetraacetic acid (EDTA) in calcium-magnesium-free Hank's balanced salt solution (CMF HBSS) (Gibco-BRL Life Technologies, Paisley, U.K.), pH 7.4 was prepared immediately before experiments.

Anaesthetised mice were killed by careful cervical dislocation; previous experiments showed this step to be critical in order to prevent damage to the aorta or other great vessels, which would inhibit perfusion. The abdomen was then opened, and a 10 -

15 cm length of proximal jejunum flushed free of intestinal contents using warm CMF HBSS. The thorax was opened and, after sectioning the caudal vena cava at the diaphragm, mice were perfused at a rate of approximately 10 ml/min via the left ventricle with 90 ml of 24 mM EDTA in CMF HBSS at 37°C using a syringe driver (Harvard Apparatus Ltd., Edenbridge, U.K.). Following perfusion, a length of jejunum was removed and divided into 2 segments, which were tied at one end onto a thin metal rod and everted. The epithelium was then released from the underlying tissue by rapid manual rotation of the gut segment in ice cold CMF HBSS, and the epithelial fragments were allowed to settle on ice. The whole procedure for each animal was completed within 20 min.

### *2.3.3 Preparation of epithelial samples*

Exfoliated epithelium in a minimum volume of CMF HBSS was removed into TRI reagent (Sigma, Poole, Dorset, U.K.) for use in RT-PCR, and vortexed for 1 min before storage at -70°C. Epithelium for use in ELISA or Western blotting was snap-frozen in dry ice and stored at -70°C. Samples of the exfoliated epithelium were placed in 4% paraformaldehyde in PBS for examination by light microscopy, in order to check the purity of the cell population collected (fig. 3.1). After 24 hours in paraformaldehyde, samples were placed in 70% alcohol before initial embedding in agar, followed by routine processing into paraffin wax. 4 µm thick sections were stained using routine haematoxylin and eosin protocols. The proportion of contaminating non-epithelial cells was assessed in haematoxylin and eosin-stained sections when 500 epithelial cells were counted.

### 2.3.4 Collection and preparation of murine whole jejunal samples

Whole proximal jejunum was collected from mice following perfusion, or from unperfused mice killed by cervical dislocation. For use in RT-PCR, jejunum was placed in RNA-*later*<sup>TM</sup> (Ambion, Austin, Texas, U.S.A.) for at least 24 h at room temperature then stored at -70°C before homogenisation, on ice, into TRI reagent. For use in Western blotting, jejunum was stored at -70°C in high-salt buffer (PBS / 0.5 M NaCl / 0.05% Tween<sub>80</sub>) with protease inhibitors (Complete<sup>TM</sup> mini EDTA-free protease inhibitor cocktail tablets, Roche, Lewes, U.K.).

## 2.4 CELL CULTURE

### 2.4.1 Murine bone marrow-derived mast cell (mBMMC) culture

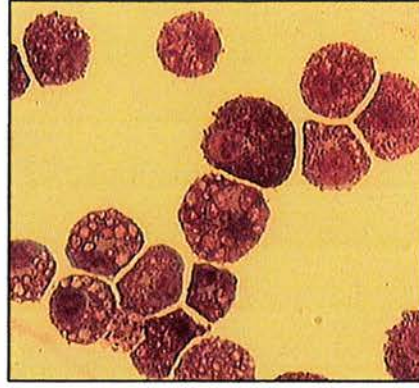
Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco-BRL. Foetal calf serum (FCS) was batch tested and purchased from Sigma. Basic culture medium (BCM) was prepared from DMEM with the addition of 10% FCS, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml of Fungizone. Complete culture medium consisted of BCM with the addition of 5 ng/ml IL-9 (R&D systems Ltd, Abingdon, U.K.), 1 ng/ml IL-3 (R&D Systems Ltd), 50 ng/ml SCF (Peprotech, London, U.K.) ± 1 ng/ml recombinant human TGF-β<sub>1</sub> (Sigma). mBMMC cultured with and without TGF-β<sub>1</sub> were termed mBMMC<sup>T+</sup> and mBMMC<sup>T-</sup>, respectively.

Mice were killed by cervical dislocation, their femurs isolated and femoral bone marrow flushed into warm BCM. In general, 5 mice were required for a sufficient yield of cells to stabilise a culture. Conditions were kept as clean as possible by

regular spraying of mice and equipment with 70% alcohol. In a sterile category 2 laminar flow hood, single cell suspensions were made by passing bone marrow three times through a 19 gauge needle followed by filtration through sterile lens tissue. The total volume of the cell suspension was made up to 50 ml with warm BCM and 2 x 10  $\mu$ l aliquots taken for counting (2.4.5). Cells were pelleted by centrifugation for 7 min at 230g, resuspended at 5 x 10<sup>5</sup> live cells/ml in complete culture medium and placed in a sterile tissue culture flask in a humid 5% CO<sub>2</sub> incubator. They were fed every 2 days, following counting, by centrifugation and resuspension at a density of 5 x 10<sup>5</sup> cells/ml in half-volume of original culture medium and half-volume of fresh complete culture medium. Cytospin preparations (2.4.6) were taken and stained with Leishman's (2.4.7) and toluidine blue (2.4.8) for enumeration of mast cells; cells used for experiments were typically 14 - 30 days old and consisted of >98% mast cells (figures 2.1 and 2.2).

Cytospin preparations were also fixed in paraformaldehyde (2.4.9) and expression of the MMC-specific chymase, mMCP-1, analysed by immunohistochemistry (2.10.3). A small proportion of mBMMC<sup>T-</sup> used for the studies described in chapter 4 expressed mMCP-1 (see 2.4.2). However, in experiments described in chapters 5 and 6, typically <2% mBMMC<sup>T-</sup> and >98% of mBMMC<sup>T+</sup> were positive for expression of mMCP-1, confirming differentiation of mBMMC<sup>T+</sup> but not mBMMC<sup>T-</sup> into MMC homologues.

(a)



(b)



(c)



**Figure 2.1. Leishman-stained cultured mast cells.**

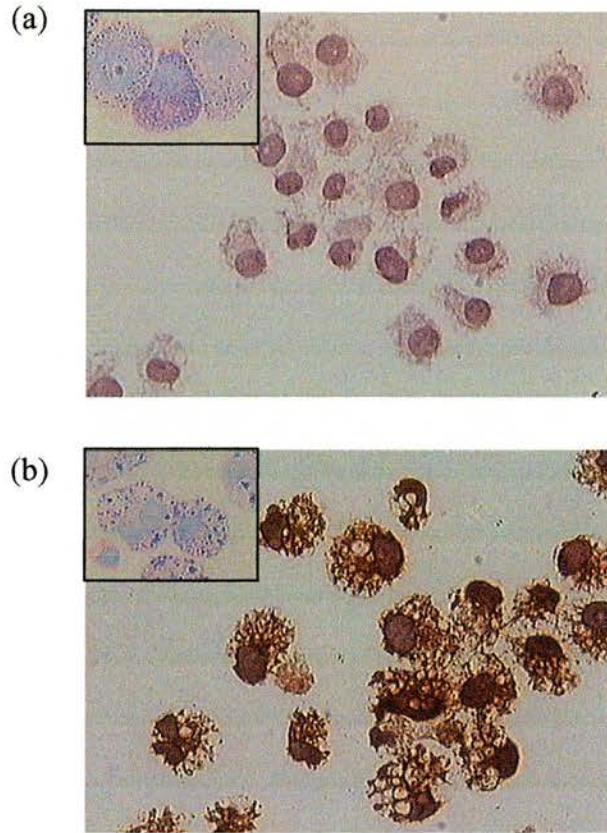
(a) mBMMC<sup>T-</sup>

(b) mBMMC<sup>T+</sup>

(c) The murine mast cell line, MC/9.

Magnification = x 40.





**Figure 2.2. Expression of mMCP-1 by mBMMC.**

Cytospin preparations of (a) mBMMC<sup>T-</sup> and (b) mBMMC<sup>T+</sup>.

Main picture - mBMMC stained by immunohistochemistry for mMCP-1, showing expression only in mBMMC<sup>T+</sup> cultured with TGF- $\beta_1$ .

Inset - toluidine blue-stained mBMMC; both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> stained with toluidine blue, which is specific for mast cells.  
(magnification = x 40).

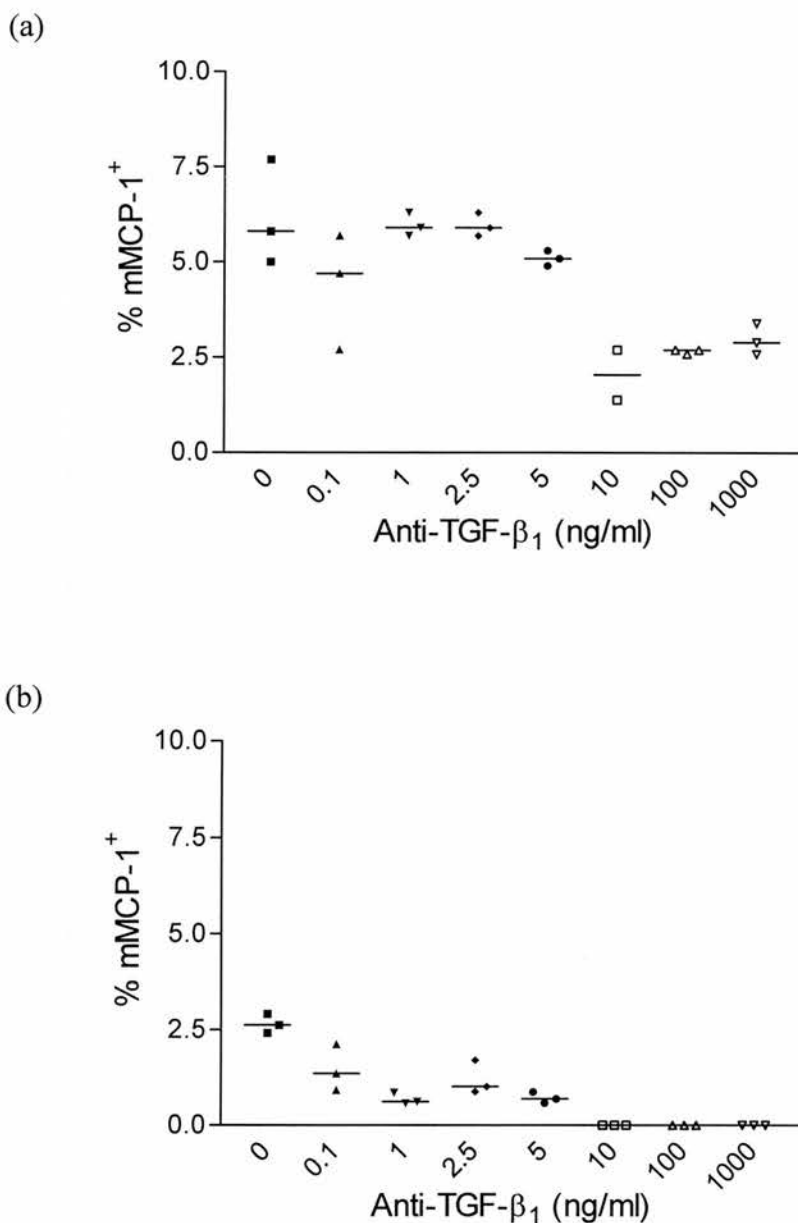
#### 2.4.2 Determination of anti-TGF- $\beta_1$ antibody concentration for mBMMC<sup>T-</sup> cultures

Previous work (Miller *et al*, 1999) showed that a proportion of mBMMC grown in culture without TGF- $\beta_1$  (mBMMC<sup>T-</sup>) may be mMCP-1<sup>+</sup>. This was suspected to be due to low levels of TGF- $\beta_1$  in the culture system from an unknown source, which could be neutralised by addition of anti-TGF- $\beta_1$  antibody. Therefore, the minimum anti-TGF- $\beta_1$  antibody concentration that would neutralise endogenous TGF- $\beta_1$  in mBMMC<sup>T-</sup> was determined by titration.

mBMMC<sup>T-</sup> cultures were set up (2.4.1), and on day 7 of culture, mBMMC<sup>T-</sup> were resuspended at  $5 \times 10^5$  cells/ml in mBMMC<sup>T-</sup> culture medium containing 0, 0.1, 1, 2.5, 5, 10, 100 or 1000 ng/ml of chicken anti-human anti-TGF- $\beta_1$  polyclonal antibody (R&D Systems). Cells were seeded in triplicate 0.5 ml volumes in 48 well tissue culture plates (Corning Costar, High Wycombe, U.K.) and cultured for 48 h after which 100  $\mu$ l of cell suspension was removed, and cytospin preparations made (2.4.6) and fixed in 4% paraformaldehyde (2.4.9) for analysis of mMCP-1 expression by immunohistochemistry (2.10.3). Statistical analysis was not performed due to insufficient numbers in each group.

After 48 h of culture, expression of mMCP-1 was low (5.8% (5.0 - 7.7%))(median (range)) in control wells containing no anti-TGF- $\beta_1$ , but numbers of mMCP-1<sup>+</sup> cells were reduced in cultures containing  $\geq 10$  ng/ml of anti-TGF- $\beta_1$  (2.7 (2.6 - 2.7)) at





**Figure 2.3. The effect of anti-TGF- $\beta_1$  antibody on spontaneous expression of mMCP-1 by mBMMC<sup>T</sup>.**

Expression of mMCP-1 after (a) - 48 h (500 cells counted) and (b) - 96 h (1000 cells counted) of culture of day 7 mBMMC<sup>T</sup> with chicken anti-human TGF- $\beta_1$  polyclonal antibody.

After 48 h of culture, numbers of mMCP-1<sup>+</sup> cells were reduced in cultures containing  $\geq 10$  ng/ml of antibody, and after 96 h there were no mMCP-1<sup>+</sup> cells in cultures containing  $\geq 10$  ng/ml antibody.

Results are expressed as median (range),  $n=3$  for all groups except 10 ng/ml anti-TGF- $\beta_1$ , 48 h culture, where  $n=2$

10 ng/ml TGF- $\beta_1$  (figure 2.3a). Because of this small difference in expression, cultures were continued for a further 48 h and the process repeated. After 96 h of culture, there were no mMCP-1<sup>+</sup> cells in wells containing  $\geq 10$  ng/ml of anti-TGF- $\beta_1$  (figure 2.3b). On the basis of the above results, 10 ng/ml of anti-TGF- $\beta_1$  antibody was included from day 10 onwards in the mBMMC<sup>T</sup> culture used for experiment 4.2.

#### *2.4.3 MC/9 cell culture*

MC/9 cells were obtained from the American Type Culture Collection (LGC-ATCC Teddington, U.K.) and cultured as instructed. DMEM was obtained from ATCC and supplemented with 6  $\mu$ g/ml folic acid (Sigma), 0.05 mM 2-mercaptoethanol (Sigma) and 2 mM glutamine (Gibco-BRL). 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Gibco-BRL) were also included. MC/9 culture medium consisted of the above, with the addition of 10% Rat T-stim® (BD Pharmingen, Oxford, U.K.) and 10% FCS (Sigma). Cells were fed every 2 days, following counting, by centrifugation and resuspension at  $2 - 5 \times 10^5$  cells/ml in half-original medium and half-fresh medium.

#### *2.4.4 Culture of CMT-93 murine intestinal epithelial cells*

The epithelial cell line CMT-93, derived from a mouse rectal carcinoma, was a gift from Dr. P.J. Kilshaw, Babraham Institute, Cambridge, U.K. Cells were seeded at  $1 - 3 \times 10^4$ /ml in Dulbecco's Minimum Essential Medium (Gibco-BRL) with 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin (Sigma) and 10% foetal calf serum (Serotec, Kidlington U.K.) and grown to confluence before harvesting into TRI reagent (2.4.10).

#### *2.4.5 Cell counting*

Cells were counted in an improved Neubauer haemocytometer. For total WBC counts, a 10 µl aliquot of cell suspension was diluted 50:50 in white blood cell counting fluid (0.01% gentian violet in 3% acetic acid in deionised water). For live/dead cell counts, an aliquot of cell suspension was diluted 50:50 in 0.2% nigrosin, and viability assessed based on nigrosin exclusion.

#### *2.4.6 Cytospin preparations*

100 µl of cells suspended at  $5 \times 10^5$  cells/ml were centrifuged for 5 min at 40g onto a glass slide in a Shandon Cytospin 2 cytocentrifuge (Southern Instruments, Runcorn, U.K.). Preparations were air-dried prior to fixation and staining.

#### *2.4.7 Leishman staining of cytopsin preparations*

Air-dried cytopsin preparations were stained with 100% Leishman's solution (Fisher Scientific, Loughborough, U.K.) for 2 min followed by 50% Leishman's solution for 6 min (by adding an approximately equal volume of deionised water to the slide). Slides were then rinsed in tap water, air dried and mounted in DPX (BDH).

#### *2.4.8 Toluidine blue staining of cytopsin preparations*

Air-dried cytopsin preparations were first fixed for 5 min in Carnoy's fixative, then rinsed in deionised water and stained overnight in 0.5% Toluidine Blue (Fisons, Loughborough, U.K.) in 0.5 M HCl. Slides were then rinsed in deionised water and counterstained briefly (1 - 2 sec) in 0.1% eosin in 70% ethanol.

#### *2.4.9 Paraformaldehyde fixation of cytospin preparations*

Air-dried cytospin preparations were fixed for 10 min using 4% paraformaldehyde. They were then rinsed with deionised water and stored in 70% ethanol at 4°C before detection of mMCP-1 expression by immunohistochemistry (2.10.3).

#### *2.4.10 Collection of cultured mBMMC and CMT-93 intestinal epithelial cells for RNA extraction*

Cultured mast cells were collected into TRI reagent and RNA extracted for use in RT-PCR.  $0.5 \times 10^6$  -  $1 \times 10^7$  cells were centrifuged for 7 min at 230g and the resultant pellet vortexed for 1 min into TRI reagent and stored at -70°C before use.

Cultured CMT-93 epithelial cells were also collected into TRI reagent. Culture medium was decanted from cells adherent to the flask and a cell scraper used to collect cells into 2 ml of TRI reagent per 75 cm<sup>2</sup> flask. RNA in TRI reagent was then aspirated, vortexed and stored at -70°C.

### 2.5 REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

#### *2.5.1 Preparation of RNA*

Total RNA was recovered from samples in TRI reagent by chloroform extraction and precipitation according to the manufacturer's instructions. RNA quality and quantity was then determined by measurement of absorbance at 260/280 nm in a Beckman DU 650 spectrophotometer. Where necessary, RNA was further checked for degradation by denaturing gel electrophoresis (2.5.2).

Some RNA samples were found to be contaminated with genomic DNA, therefore samples were DNase-treated using a DNA-free™ kit (Ambion, Austin, Texas, U.S.A.). 50 µl RNA samples were incubated for 40 min at 37°C with 2 µl (4 U) of DNase I and 5 µl of 10 x DNase buffer. Samples were then incubated for 2 min at room temperature with 5 µl of DNase inactivation reagent, flicking once during incubation to disperse the reagent. The inactivation reagent was pelleted to remove it from suspension, by centrifugation at 10000g for 1 min. RNA samples were then re-quantified by spectrophotometry as before.

### *2.5.2 Denaturing gel electrophoresis*

Under RNase free conditions, a 1 x MOPS, 1% agarose gel mixture was made and 2 ml of 37% formaldehyde added before pouring. 6 µl RNA samples were incubated at 65°C for 10 min with 12.5 µl of deionised formamide (Sigma), 2.5 µl of 10 x MOPS and 4 µl of 37% formaldehyde. 2.5 µl of Northern running dye (50% glycerol / 0.1 mg/ml bromophenol blue in RNase-free water) and 0.5 µl of ethidium bromide (0.5 mg/ml) was then added to each sample, and 20 µl loaded onto the gel. Samples were electrophoresed in a 1 x MOPS running buffer for 1 h at 70 V. An image was visualised and recorded under UV light using a charge-coupled device (CCD) camera linked to an image processor (Appligene, Watford, U.K.).

### *2.5.3 Preparation of cDNA*

1 µg of each RNA sample was reverse-transcribed in a 20 µl volume using a Promega Reverse Transcription (RT) kit (Promega, Southampton, U.K.). Reactions contained 1 x RT buffer (10 mM Tris-HCl / 50 mM KCl / 0.1% Triton X-100, pH 8.8), 1 mol/l of each dNTP, 20 U of RNase inhibitor, 0.5 µg of oligo dT primer, 5

mol/l of  $\text{MgCl}_2$  and 15 U of avian myoblastoma virus (AMV) reverse transcriptase. Incubation was for 1 h at 42°C followed by 5 min at 99°C to inactivate the enzyme.

#### 2.5.4 Primers and probes

If available, published primer sequences were used, otherwise primers were designed using RNA and genomic DNA sequences from Genbank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The internet primer design software programmes "Genefischer" ([www.genefisher.de](http://www.genefisher.de)) and "Netprimer" ([www.premierbiosoft.com](http://www.premierbiosoft.com)) were employed to aid design of suitable primers; all primers were intron spanning where possible. Oligonucleotide probes for use in Southern blotting were also designed as above by examination of PCR product sequences derived from Genbank. Oligos for primers and probes were obtained from Cruachem (Paisley, U.K.) or VHBio (Sunderland, U.K.); sequences of primers and probes are shown in tables 2.1 - 2.4.

#### 2.5.5 Polymerase chain reaction (PCR)

PCRs were initially set up according to a standard method (below) and optimised as necessary (2.5.7). Reactions were set up in 0.2 ml thin-walled PCR tubes (Perkin Elmer MicroAmp) to a final volume of 50  $\mu\text{l}$  containing 1  $\mu\text{l}$  (1/20<sup>th</sup> vol. of RT reaction) of cDNA, 50  $\mu\text{mol/l}$  of each dNTP (Promega, Southampton, U.K.), 200 nmol/l of each primer, 1 x Roche PCR buffer (50 mM KCl / 10 mM Tris-Cl / 1.5 mM  $\text{MgCl}_2$ ) and 2.5 U of *taq* polymerase (Roche, Lewes, U.K.). Cycle parameters were - Initial denaturation, 30 s at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, and a final extension period of 7 min at 72°C. For each sample, a negative control was set up containing RNA only (no cDNA) to rule out contamination with genomic DNA, and each PCR experiment included a negative

Gene	Oligo sequence	Product Size (BP)	Tann. (°C)	Mg <sup>2+</sup> (mM)	pH
GAPDH	S-5' GAAGGGCTCATGACCACAGTCCATG A-5' TGTTGCTGTAGCCGTATTCATTGTC	454	55	1.5	8.3
MCP-1 <sup>1</sup>	S-5' GGAAAAATGGATCCACACCTTGC A-5' TCTCTTCCTCCACCACCATGCAG P-5' CCAGTGTGACAGTGAAGTAGTGTG	582	60	1.5	8.6
SCF <sup>2</sup>	S-5' AACTTGGATTATCACTTGCAT A-5' CAATGCCATGGCTGTCCATTG P-5' TGACCTCGTGTATGC	562 + 646 †	55	1.5	8.3
TGF-β <sub>1</sub> <sup>3</sup>	S-5' CGGGGCGACCTGGGACCATCCATCAG A-5' CTGCTGCACCTTGGGCTTGCGACCCAC P-5' CTGGAAAGGGCCCAGCACCT	406	55	1.5	8.3
RANTES <sup>1</sup>	S-5' TCATCCTCACTGCAGCCGCCC A-5' CTCTATCCTAGCTCATCTCCAAA P-5' CCTCTCCCTCGCGCTGCCTCGTG	254	55	1.5	8.3
MIP-1α <sup>1</sup>	S-5' GAAGAGTCCCTCGATGTGGCTA A-5' CCCTTTTCTGTTCTGCTGACAAG P-5' CCCC GACTGCCTGCTGCTTCTCC	462	55	1.5	8.3
Fractalkine <sup>4</sup>	S-5' TGGTCCAGAGCTGGCAATAA A-5' TGGCTTCCTCACTCTCAGGA P-5' GTGTAGGGAAGTGTGCATGTGTG	816	50	1.5	8.3
TECK <sup>5</sup>	S-5' TTTGAAGACTGCTGCCTGG A-5' GTCTTCTTCCTAACAAGCC P-5' GAGCGCCTCAGACTCTCAGACTG	742	55	1.5	8.3
mMCP-1 <sup>3</sup>	S-5' GGAAAACTGGAGAGAAAGAACCTAC A-5' GACAGCTGGGGACAGAATGGGG	460	63	1.5	8.3

**Table 2.1. Primer and probe sequences, and reaction conditions used for experiments 3.2 and 3.3.**

1 µl of cDNA was amplified for 35 cycles, except for measurement of MCP-1 expression by semi-quantitative PCR, when 1 µl of cDNA was amplified for 22 cycles using primers specific for GAPDH, and 10 µl of cDNA was amplified for 34 cycles using primers specific for MCP-1.

S - sense primer, A - antisense primer, P - probe. Tann. - annealing temperature. + - secreted form, † - transmembrane form of SCF. All primers were published sequences (references shown in footnotes) except those for GAPDH, which were from Stratagene (Amsterdam, The Netherlands).

<sup>1</sup> Rollo *et al*, 1999

<sup>2</sup> Mitsunari *et al*, 1999

<sup>3</sup> Miller *et al*, 1999

<sup>4</sup> Kanazawa *et al*, 1999

<sup>5</sup> Wurbel *et al*, 2000

Gene	Oligo Sequence	Product Size (BP)	Tann. (°C)	Mg <sup>2+</sup> (mM)	pH	Cycle No.
GAPDH	S-5' GAAGGGCTCATGACCACAGTCCATG A-5' TGTTGCTGTAGCCGTATTCATTGTC	454	55	1.5	8.3	35 22*
mMCP-1 <sup>1</sup>	S-5' GGAAAACTGGAGAGAAAGAACCTAC A-5' GACAGCTGGGGACAGAATGGGG	460	63	1.5	8.3	35
CXCR2 <sup>2</sup>	S-5' ACCAGTTATGCTGTGGTTGTA A-5' CAAACGGGATGTATGTTACC P-5' CTGTTCTTTGCCCTGACCTT	483	50	1.5	8.3	35
CXCR4 <sup>3</sup>	S-5' TCTTAGGGACTTGTGCATGTGTG A-5' CGTCGGCAAAGATGAAGTCAGG P-5' CGCCTGCACCTGTCAGTGGC	493	55	1.0	9.3	35

**Table 2.2. Primer and probe sequences, and reaction conditions used for RT-PCR in experiments 4.2, 4.3 and 4.4.**

1 µl of cDNA was amplified as indicated; GAPDH was amplified for 22 cycles in semi-quantitative RT-PCR (\*).

S - sense primer, A - antisense primer, P - probe. Tann. - annealing temperature. All primers were published sequences (references shown in footnotes), except those for GAPDH, which were from Stratagene (Amsterdam, The Netherlands).

<sup>1</sup> Miller *et al*, 1999

<sup>2</sup> Fischer *et al*, 2000

<sup>3</sup> Papadopoulos *et al*, 2000



Gene	Oligo sequence	Product size (BP)	Tann. (°C)	Mg <sup>2+</sup> (mM)	pH	Cycle no.	Ref.
α3	S-5'-ATT GAC TCA GAG CTG GTG GAG GAG A-5'-TAC TTG GGC ATA ATC CGG TAG TAG P-5' - GCT GGG TTG CTG CTG CTG GG S-5'- TTG CTG GAC TGT GGT GAA GAC A-5'- CTG GTT GAT GAG CTG GTA GAC P-5' - CAG TCT CTG GGG TGG CCT TC S-5'- GAG GAA TAT TCC AAA CTG AAC TAC A-5'- GGA ATG CTG TCA TCG TAC CTA GAG P-5' - CGA TGC CAC CTA TCA CAA GG S-5'- CCG CCG GTG CCA GCC CAT TGA G A-5'- GCT ACC AGG ACC ACC GAG AAG T P-5'-CTG TGC TCC ATT GTA CCA CT	α3A 305 α3B 161	63	2.0	8.3	35	Anderson <i>et al</i> , 1999
α5	S-5'- TTG CTG GAC TGT GGT GAA GAC A-5'- CTG GTT GAT GAG CTG GTA GAC P-5' - CAG TCT CTG GGG TGG CCT TC S-5'- GAG GAA TAT TCC AAA CTG AAC TAC A-5'- GGA ATG CTG TCA TCG TAC CTA GAG P-5' - CGA TGC CAC CTA TCA CAA GG S-5'- CCG CCG GTG CCA GCC CAT TGA G A-5'- GCT ACC AGG ACC ACC GAG AAG T P-5'-CTG TGC TCC ATT GTA CCA CT	582	55	1.5	8.3	32	(Dastyeh <i>et al</i> , 2001)
α6	S-5'- GAG GAA TAT TCC AAA CTG AAC TAC A-5'- GGA ATG CTG TCA TCG TAC CTA GAG P-5' - CGA TGC CAC CTA TCA CAA GG S-5'- CCG CCG GTG CCA GCC CAT TGA G A-5'- GCT ACC AGG ACC ACC GAG AAG T P-5'-CTG TGC TCC ATT GTA CCA CT	α6A 399 α6B 269	55	2.5	8.3	35	Anderson <i>et al</i> , 1999
αv	S-5'- CCG CCG GTG CCA GCC CAT TGA G A-5'- GCT ACC AGG ACC ACC GAG AAG T P-5'-CTG TGC TCC ATT GTA CCA CT	337	55	1.5	8.3	32	Illera <i>et al</i> , 2000
α7	S-5'- CAC CGG ATG TCC ATC AGG AC A-5'- GTT GCT CAG AGA TGC ATC C P-5' - CTG GTA CTG CTG CTG TGG AA S-5'- GGC AAC AAT GAA GCT ATC GT A-5'- CCC TCA TAC TTC GGA TTG AC P-5' - TTG GGA TGA TGT CGG GAC	α7A 480 α7B 366	55	1.5	8.3	35	Ziober <i>et al</i> , 1993
β1	S-5'- GGC AAC AAT GAA GCT ATC GT A-5'- CCC TCA TAC TTC GGA TTG AC P-5' - TTG GGA TGA TGT CGG GAC	β1A 266 β1D 348	55	1.0	8.3	30	Anderson <i>et al</i> , 1999
β3	S-5'- CTG GTG TTT ACC ACT GAT GCC AAG A-5'- TGT TGA GGC AGG TGG CAT TGA AGG P-5' - CCA CAG TGG GAG TCC TGT CT S-5'- CTG AAG TTT GCT GCT GCC ACA A-5'- GCT GGA TGG GCA CAG AGG GAA P-5' - ATG CCT GAT CTT TAC TTC TA	393	60	2.5	8.9	38	Illera <i>et al</i> , 2000
LBP	S-5'- CTG AAG TTT GCT GCT GCC ACA A-5'- GCT GGA TGG GCA CAG AGG GAA P-5' - ATG CCT GAT CTT TAC TTC TA	520	55	1.0	8.3	26	
GAPDH	S-5'-GAAGGGCTCATGACACAGTCCATG A-5'- TGTGCTGTAGCCGTAATTCATTGTC	454	55	1.5	8.3	20	

**Table 2.3. Primer and probe sequences and conditions used for semi-quantitative RT-PCR analysis of expression of integrins and LBP by mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> in experiment 6.2.**

S - sense primer, A - antisense primer, P - probe. Tann. - annealing temperature. All primers were published sequences (references shown), except GAPDH primers, which were from Stratagene (Amsterdam, The Netherlands).

gene	Oligo sequence	Product size (BP)	Tann. (°C)	Mg <sup>2+</sup> (mM)	pH	Cycle no.	Ref.
X1	S-5' CTATCCTTGGCGAGAATGAC A-5' GCCAGGGGTGGAGCTCTG	220	55	1.5	8.3	35	Ziöber <i>et al</i> , 1993
X2	S-5' CTATCCTTGGCGAGAATGAC A-5' GTGACCAACATTGATAGCTC	200	55	1.5	8.3	35	Ziöber <i>et al</i> , 1993
GAPDH	S-5' GAAGGGCTCATGACCACAGTCCATG A-5' TGTGCTGTAGCCGTATTCAATTGTC	454	55	1.5	8.3	35	

**Table 2.4. Primers and conditions used for RT-PCR analysis of expression of  $\alpha 7X1$  and  $\alpha 7X2$  in experiment 6.10.**

Conditions shown for X2 are those used for initial investigations, but no signal was obtained in positive control heart tissues after extensive optimisation of the protocol.

S - sense primer, A - antisense primer. Tann. - annealing temperature. All primers were published sequences (references shown), except GAPDH primers, which were from Stratagene (Amsterdam, The Netherlands).

control omitting cDNA. Final reaction conditions used for each primer are shown in tables 2.1 - 2.4.

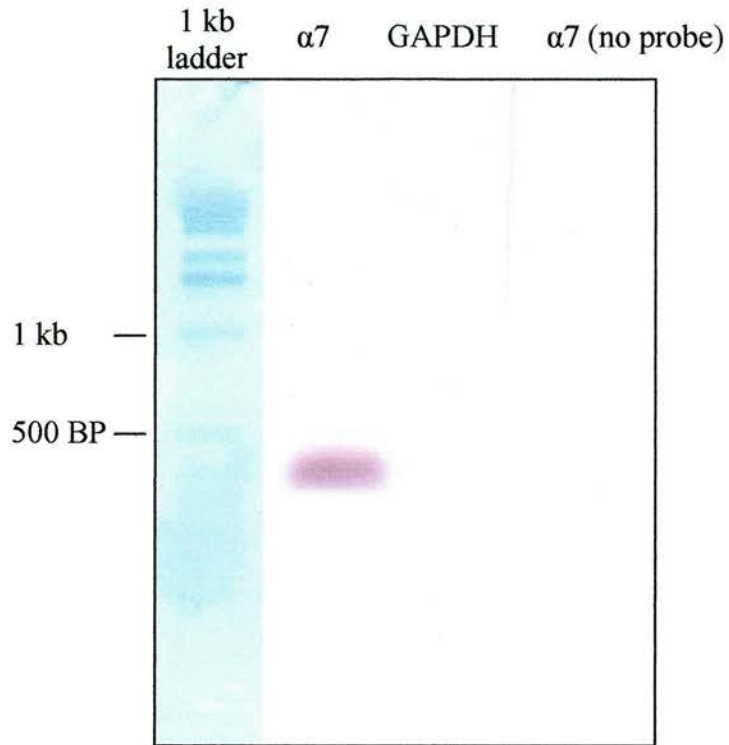
PCR products were separated on a 1.4 % agarose gel containing 0.5 µg/ml ethidium bromide and visualised and recorded under UV light using a Kodak Image Station 440cf imaging system.

#### *2.5.6 Confirmation of product identity by Southern blotting*

PCR product identities were confirmed by Southern blotting and detection using an internal oligonucleotide probe. Each probe was also incubated with the PCR product from amplification of an unrelated gene (usually GAPDH) to check probe specificity, and samples were hybridised without probe to confirm the specificity of antibody binding to digoxigenin (DIG)-labelled probe.

Oligonucleotide probes were DIG-labelled using a Roche 3' end labelling kit, according to the manufacturer's instructions, and labelled probe diluted into 10 ml of "Rapidhyb" hybridisation buffer (Roche), resulting in a final concentration of labelled probe of 10 pmol/ml.

Representative PCR products for each gene were separated on a 1.4 % agarose gel, denatured for 30 min in 2 changes of denaturing buffer (1.5 M NaCl / 0.5 M NaOH) and neutralised for 40 min in 2 changes of neutralising buffer (1.5 M NaCl / 0.5 M Tris-HCl / 1 mM EDTA). DNA was then transferred overnight to a nylon membrane (Hybond N+, Amersham, U.K.) by capillary blotting. Following blotting, the membrane was washed in 6 x saline-sodium citrate (SSC) and nucleic acid was



**Figure 2.4. Confirmation of PCR product identity by Southern blotting.**

$\alpha 7$  integrin is used as an example.

Digoxigenin-labelled  $\alpha 7$  oligonucleotide probe hybridised to the 266 BP  $\alpha 7$ B PCR product, whereas the probe did not hybridise to the negative control, GAPDH. Also, no  $\alpha 7$  integrin PCR product was detected when oligonucleotide probe was omitted ( $\alpha 7$  no probe).

fixed by two 12 s exposures to UV light. It was then prehybridised for 30 min at 40°C in hybridisation buffer, and hybridised with probes for 3 h at 40°C before washing twice for 5 min in 2 x SSC / 0.1% sodium dodecylsulphate (SDS) followed by two 15 min washes in 0.1 x SSC / 0.1% SDS. Membranes were then rinsed briefly in maleic acid buffer and blocked by incubating for 60 min at room temperature or overnight at 4°C in blocking buffer (5 g Roche blocking reagent dissolved in 500 ml maleic acid buffer). Hybridised probes were labelled by incubation for 30 min with anti-DIG alkaline phosphatase conjugate (Roche) diluted 1:5000 in blocking buffer, after which unlabelled probe was washed away by two 15 min washes in maleic acid 0.3 % Tween<sub>20</sub>.

Alkaline phosphatase - labelled probes were detected using 5-bromo-4-chloro-3-indoyl phosphate / nitroblue tetrazolium (BCIP/NBT) (Sigma-fast tablets) or chemiluminescent detection with CDP-star (Roche), and an image acquired using up to ten 1 min captures on a Kodak Image station 440cf imaging system. An example of PCR product confirmation by Southern blotting is shown in fig. 2.4.

#### *2.5.7 Optimisation of PCRs*

Where necessary, PCRs were optimised as follows so that a product of the appropriate size was obtained against a background of minimal non-specific PCR products. Cycle parameters were as described, but with alterations in annealing temperature, Mg<sup>2+</sup> concentration and pH as detailed below.

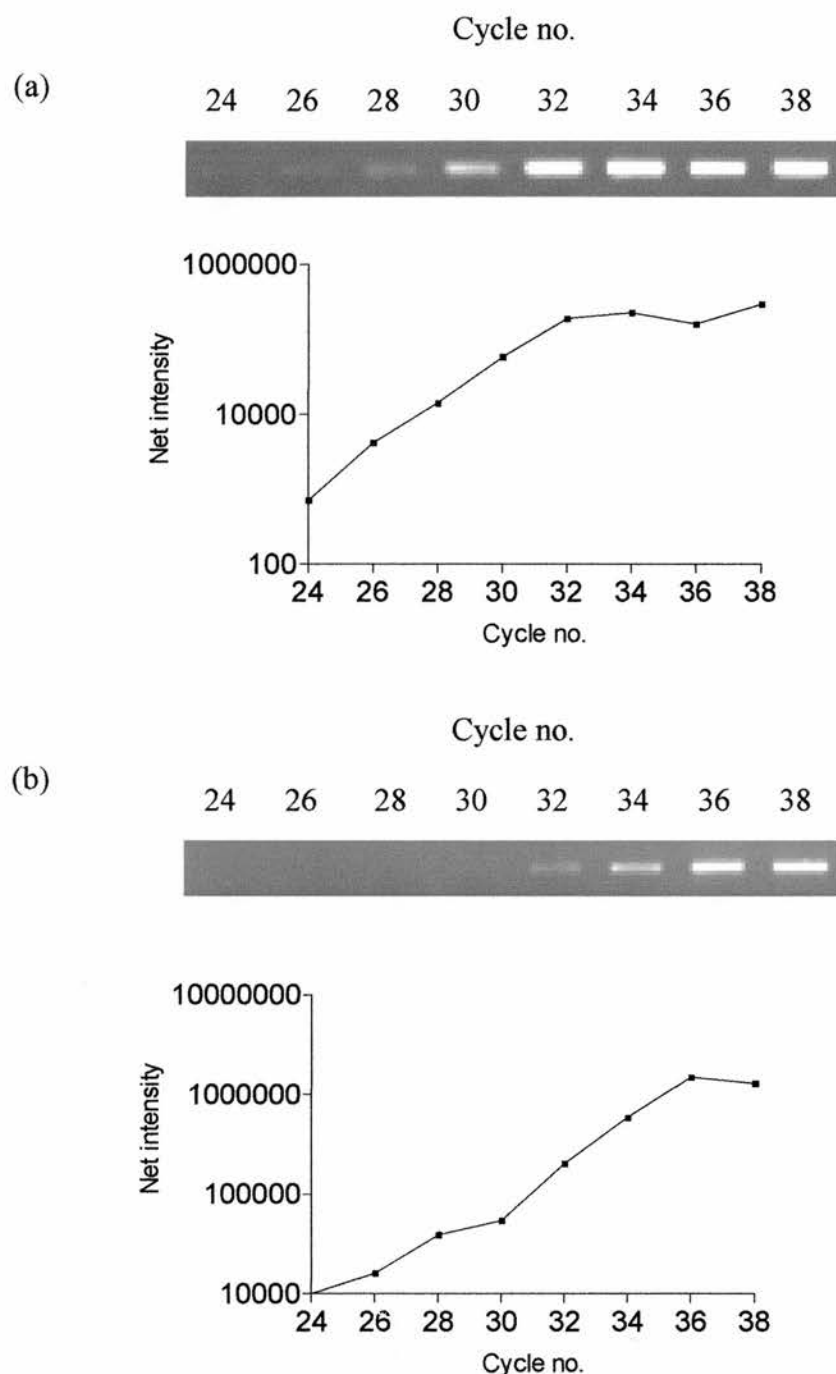
The initial annealing temperature chosen was 5 - 10°C below the melting temperature of both primers, and primers were then optimised using a Roche PCR

optimisation kit for 35 cycles of PCR. PCRs were first optimised with respect to  $\text{MgCl}_2$  concentration, followed by pH and annealing temperature.

Primers were initially tested using buffers of pH 8.3 at  $\text{Mg Cl}_2$  concentrations of 1.0, 1.5, 2.0 and 2.5 mM, then if required they were tested at the optimum  $\text{MgCl}_2$  concentration at pH 8.3, 8.6, 8.9 and 9.2. Finally, as increasing annealing temperature reduces formation of non-specific PCR products, the annealing temperature was altered as necessary. If no signal was obtained during initial stages, annealing temperature was lowered and primers tested using all available buffers.

#### *2.5.8 Semi-quantitative PCR*

In order to make PCR semi-quantitative, cycle numbers were optimised so that the amount of PCR product would be proportional to the amount of RNA in the initial sample. Following optimisation of PCRs, reactions were set up to amplify representative samples for a range of cycle numbers, and PCR products separated on agarose gel as described (2.5.5). The image was then acquired using multiple captures, to avoid saturation of the image acquisition system, and band intensities quantified using Kodak 2D Image software. Net band intensities were then plotted against cycle number on a logarithmic scale, and a cycle number chosen within the linear range of the graph which would result in a detectable signal for all samples (figure 2.5). Expression of the house-keeping gene GAPDH was also optimised as above for use as a control for the amount of cDNA in each sample, and relative expression for each sample calculated as the ratio of signal intensity of the gene investigated to GAPDH.



**Figure 2.5. Optimisation of cycle number for semi-quantitative RT-PCR.**

A representative sample was amplified for a range of cycle numbers, and net intensity of resultant signals measured using Kodak 1D software. This was plotted against cycle number on a logarithmic scale. A cycle number was then chosen for use in RT-PCR, which would result in a detectable signal whilst remaining in the log phase of the reaction.

(a)  $\beta 1$  integrin - 30 cycles chosen for semi-quantitative RT-PCR (b)  $\alpha 6$  integrin - 35 cycles chosen for semi-quantitative RT-PCR.

When comparing expression of a gene for which levels varied widely between samples, it was not possible to detect low levels of expression without some degree of saturation in samples where levels were high. In this case, reactions were optimised to detect low expression and it was accepted that saturation of the reaction where the gene was highly expressed would result in underestimation of expression. Reactions optimised in this way included analysis of expression of mMCP-1, CXCR2 and CXCR4 during mBMMC differentiation (4.3) and comparison of expression of  $\alpha 7$  integrin in mBMMC cultured with and without TGF- $\beta_1$  (6.2). Reaction conditions used for semi-quantitative RT-PCR are shown in tables 2.1 - 2.3.

#### *2.5.9 Extraction and amplification of genomic DNA*

PCR was performed on mouse genomic DNA using the test primers to confirm that bands of the correct size would only be obtained from amplification of cDNA. DNA was extracted from mouse tails using a DNeasy kit (Qiagen, Crawley, U.K.). Briefly, the mouse tail was digested for 4 h at 55°C in 180  $\mu$ l of tissue lysis buffer containing 20  $\mu$ l of Proteinase K. The resulting suspension was vortexed then DNA extracted using the DNeasy column according to the manufacturer's instructions. The DNA solution was quantified using spectrophotometry as described previously for RNA. 100 ng of DNA was amplified using reaction conditions described for individual primers.

#### **2.6 MULTIPLEX PCR (mPCR)**

Expression of mRNA for chemokine receptors expressed by mBMMC was detected using CytoXpress<sup>TM</sup> multiplex (m) PCR kits (Biosource, Nivelles, Belgium). mPCR



can be problematic since the presence of many PCR primers in a single reaction can lead to increased formation of mis-primed PCR products and "primer dimers", and of enhanced amplification of shorter DNA fragments. The primer pairs and buffers in these kits have been optimised to minimise these problems and provide semi-quantitative amplification of each gene. Additionally, since the amplification efficiencies are equivalent, band intensities are proportional to the relative concentration of target cDNA in the sample, so that expression levels of each receptor can be compared to others in the same kit.

### 2.6.1 Basic CytoXpress™ mPCR method

CytoXpress™ CCR set1 and CCR set 2 mPCR kits amplified chemokine receptor genes as detailed in table 2.5. Kits contained optimised primer mixtures, buffers, dNTP and positive control cDNA.

CCR Set 1		CCR Set 2	
GAPDH	532	GAPDH	532
CCR1	363	CCR6	306
CCR2	161	CCR7	204
CCR3	315	CCR8	429
CCR4	282	CCR9	255
CCR5	161	CX3CR1	349

**Table 2.5. Primers and expected product sizes (base pairs) in Biosource CytoXpress™ mPCR kits.**

RNA was extracted from samples in TRI reagent, DNase-treated and reverse-transcribed (2.5.1 and 2.5.3), and 5 µl used in mPCR reactions according to the manufacturer's instructions. 50 µl mPCRs contained 5 µl of mPCR buffer, 5 µl of mPCR primers, 4 µl of dNTP (3.12 mM), 5 µl of positive control or test cDNA, 0.5 µl of *taq* polymerase (5 U/µl) and 30.5 µl of RNase-free water. cDNA was amplified

using a "hot-start" method starting with a 1 min initial denaturation at 96°C, followed by 3 cycles of 1 min at 96°C, 4 min at 58°C. This was followed by 28 cycles of 1 min at 94°C, 1 min at 58°C, and an elongation step of 10 min at 70°C. PCR products were separated on a 2% agarose gel containing 0.5 µg/ml of ethidium bromide, and visualised and recorded under UV light using a Kodak Image Station 440cf imaging system. Where indicated, relative expression of genes investigated using CytoXpress™ kits was calculated as the ratio of intensities of each PCR product to intensity of the GAPDH for the same sample. Though the multiplex kits were optimised for semi-quantitative RT-PCR, the manufacturers advised that expression could be compared to an external GAPDH analysed in the same samples by RT-PCR if the internal GAPDHs appeared saturated. However, GAPDH signals varied between samples and did not appear saturated; and variations correlated with differences in signals within groups, but did not correlate well with signals from external GAPDH, possibly due to differences in loading or amplification efficiencies. Therefore, the internal GAPDH was decided to be the most appropriate control.

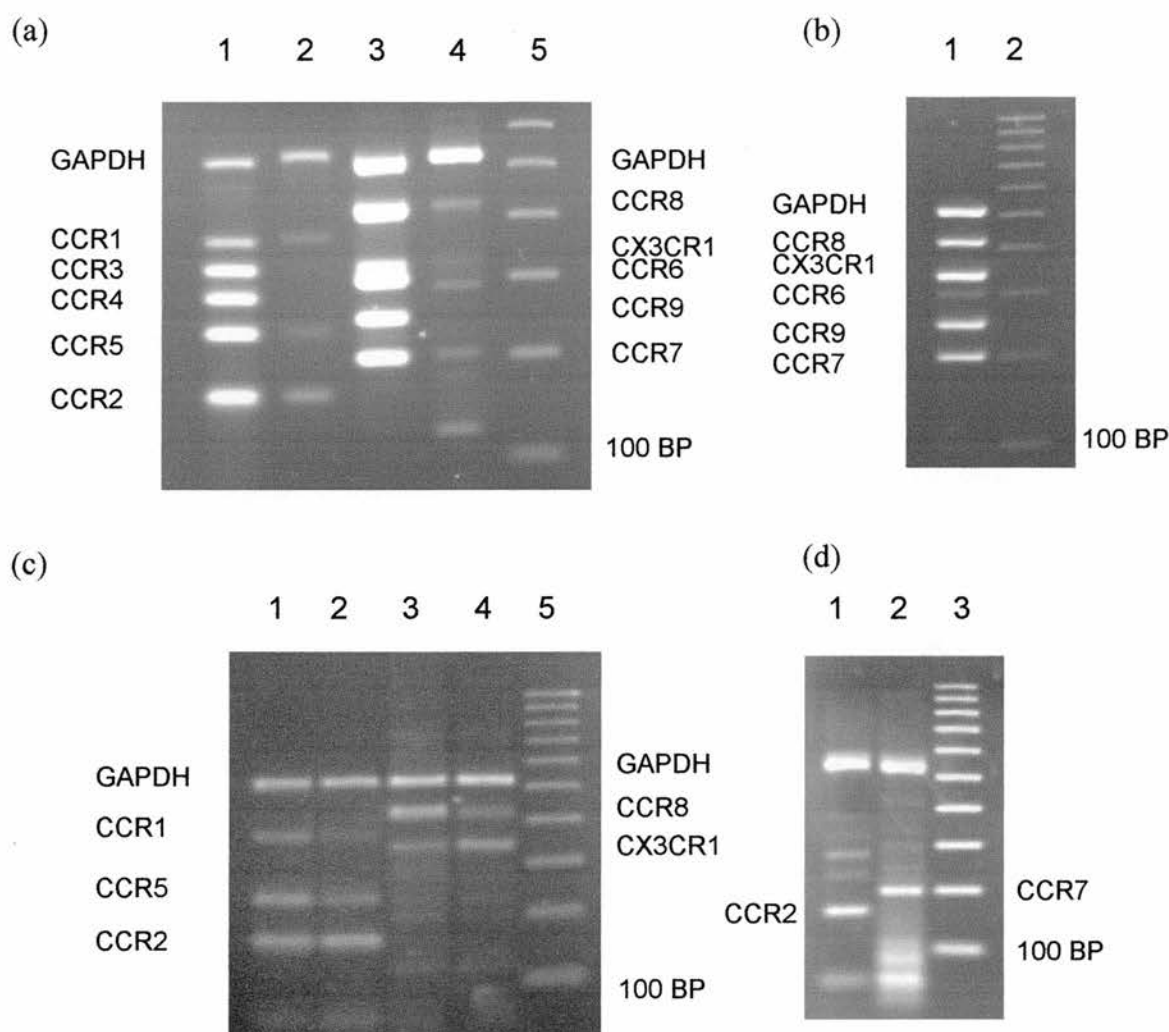
### *2.6.2 Optimisation of mPCR kits*

To ensure recommended reaction conditions would be compatible with the PCR machine used, kits were first tested using the above method with the positive control provided. This resulted in clear bands of the expected size, with the exception of CX3CR1, which ran lower than the expected size of 349 BP (figure 2.6a). The reason for this was not resolved, though the CCR set 1 kit originally provided was used for experiment 4.2, and a replacement kit was eventually provided by Biosource (figure. 2.6b), and used for experiment 4.3. The CCR6 PCR product from amplification of positive control cDNA using the replacement CCR set 2 kit was less

intense than other bands (figure 2.6b). This suggested less efficient amplification of CCR6 by this kit but as this receptor was of secondary importance to CX3CR1, which had previously been shown to be expressed by mast cells, the replacement kit was deemed adequate for our studies.

CytoXpress™ kits were also tested using 5 µl of day 14 mBMMC<sup>T+</sup> cDNA synthesised using standard method 2.5.3, alongside positive control samples, to determine if this amount of cDNA would result in a strong GAPDH signal and detection of any chemokine receptor signals. PCR of mast cell samples resulted in an adequate house-keeping gene signal and amplification of several bands, a result which was repeatable (figure 2.6a).

Biosource suggested removal of RT enzyme from cDNA before PCR, since it may inhibit the reaction. We therefore compared results of mPCR amplification, using both kits, of a non-extracted cDNA sample and the same cDNA sample after extraction using a "high-pure" spin-column (Roche), according to the manufacturer's instructions. Two 20 µl cDNA preparations were made from a day 14 mBMMC<sup>T+</sup> RNA sample. One 20 µl cDNA preparation was then extracted using the high-pure kit, and the resultant cDNA resuspended in the minimum volume (50 µl) of Tris buffer, pH 8.0. PCR was then set up using 0.25 x volume of each cDNA preparation i.e. 5 µl of the non-extracted and 12.5 µl of extracted preparations, for each of the two kits. There was little difference between PCR products from extracted and non-extracted cDNA samples (figure 2.6c), therefore no extraction was performed in further experiments.



**Figure 2.6. Optimisation of CytoXpress™ mPCR kits.**

- (a) Testing of CCR set 1 (lanes 1 and 2) and set 2 (lanes 3 and 4) using positive control cDNA (1 and 3) or d14 mBMMC<sup>T+</sup> cDNA sample (2 and 4). This shows amplification of chemokine receptor cDNA in positive control resulting in bands of the expected size, with the exception of CX3CR1, which is running lower than the expected 349 BP. Expression of several chemokine receptors is also detected in mBMMC<sup>T+</sup>.
- (b) Replacement set 2, showing CX3CR1 band running in the correct place (349 BP).
- (c) Comparison of PCR products resulting from amplification of extracted (lanes 2 and 4) and non-extracted (lane 1 and 3) samples using CCR set 1 (lanes 1 and 2) and replacement set 2 (lanes 3 and 4). Similar expression of chemokine receptors is detected in both.
- (d) Use of mouse genomic DNA as target for set 1 (lane 1) and replacement set 2 (lane 2) showing several bands amplified strongly, especially CCR2 in set 1, and CCR7 in set 2.

Lane 5 (a), 2 (b), 5 (c) and 3 (d) = 100 BP ladder. Positive control cDNA was not included in experiments (c) and (d) due to limited availability.

Finally, manufacturer's instructions were unclear if the primers were intron-spanning, or if they amplified genomic DNA and if resulting products would be distinguishable by size, from those resulting from cDNA amplification. PCR reactions were therefore set-up, using 1 µl of a mouse-tail genomic DNA sample, prepared in method 2.5.9, as target. CytoXpress™ kits amplified mouse genomic DNA with bands of similar sizes to those resulting for amplification of cDNA (figure 2.6d).

## 2.7 GEARRAY™ ASSAY PROTOCOL

A mouse chemokine Non-rad (non-radioactive) GEMatrix™ macroarray kit (Superarray™, Cambridge Bioscience, Cambridge, U.K.) was used to compare expression of a number of chemokines in jejunal epithelium of uninfected and *N. brasiliensis* infected mice. The mouse chemokine GEMatrix™ is a nylon membrane spotted in duplicate with gene-specific cDNA fragments for 23 chemokines; their positions on the array are shown in figure 2.7. House keeping genes GAPDH and  $\beta$ -actin, and bacterial plasmid DNA are also included as controls. In the non-radioactive labelling system, RNA samples are first labelled with biotin before hybridisation with the array, and hybridised labelled probes are detected with alkaline phosphatase conjugated streptavidin and addition of a chemiluminescent substrate.

### 2.7.1 Probe synthesis

The method followed instructions provided for non-radioactive GEMatrix™ protocol; the kit components are shown in table 2.6. The integrity of each RNA sample was checked by denaturing gel electrophoresis (2.5.2), as RNA degradation was not well

## Key



Gene	GEA location
6 Ckine-ser	1, 2
Eotaxin	3, 4
Eotaxin-2	5, 6
Fractalkine	8, 9
GRO1	10, 11
I-309	12, 13
IP-10	15, 16
Lymphotactin	17, 18
MCP-1	19, 20
MCP-1	22, 23
MCP-3	24, 25
MCP-5	26, 27
MIG	29, 30
MIP-1 $\alpha$	31, 32
MIP-1 $\beta$	33, 34
MIP-1 $\gamma$	36, 37
MIP-2	38, 39
MIP-3 $\alpha$	40, 41
PF4	43, 44
RANTES	45, 46
SDF-1 $\alpha$	47, 48
SDF-2	50, 51
TECK	52, 53
B-actin	21, 28
GAPDH	35, 42, 49, 54, 55, 56
PUC18 bacterial plasmid	7, 14

**Figure 2.7. Chemokines investigated using the SuperArray™ Mouse Chemokine GEAArray™ kit.**

Nonrad-GEArray labelling kit	Nonrad-GEArray detection kit
GEAprimer mix, buffer A	GEAblocking solution
5 x non-radioactive GEAlabelling buffer BN	AP-streptavidin
10 x stop solution, buffer C	5 x washing buffer F
10 x denaturing solution, buffer D	1 x assay buffer G
2 x neutralising solution, buffer E	CDP-star™
GEAhyb hybridisation solution	
+ 2 x mouse chemokine GEArray™ membranes	

**Table 2.6. GEArray components provided in "Nonrad-GEArray" Kit.**

Reaction component	Vol. (µl)
5 x Non-radioactive-GEAlabelling buffer	16
Biotin-16-dUTP (1 mM, Roche)	8
RNase inhibitor (4 U/µl, Promega)	2
AMV reverse transcriptase (25 U/µl, Roche)	8
RNase free H <sub>2</sub> O	6
Total vol.	40

**Table 2.7. GEArray labelling mixture made up for 2 samples.**

tolerated by the GEMatrix™ system. 3.3 µg of RNA from each of 3 uninfected and 3 infected samples were then combined to make 9.9 µg of RNA from uninfected and infected epithelium, and a labelled RNA probe made from each combined RNA sample. Each 9.9 µg RNA sample was mixed with 2 µl of GEMatrix primer mix (buffer A) in a final volume of 20 µl. This was heated to 70°C for 2 min and cooled to 42°C before adding 20 µl of labelling mix (table 2.7) to each sample and incubating at 42°C for 2 h. The labelled probes were then denatured by incubation for 20 min at 68°C with 5 µl of 10 x denaturing solution; denatured probes were neutralised by a 10 min incubation with 50 µl of 2 x neutralising solution.

### *2.7.2 Hybridisation*

15 ml of GEMatrix hybridisation solution was heated to 68°C, and 100 µg/ml of heat-denatured sheared salmon sperm DNA added. Two GEMatrix membranes were then wetted with deionised water, placed in separate hybridisation bottles and pre-hybridised, shaking, for 2 h at 68°C in 10 ml of GEMatrix hybridisation solution prepared as above. Prehybridisation solution was then discarded, and the membranes incubated overnight at 68°C, shaking, with the denatured probes (100 µl) in 5 ml of GEMatrix hybridisation solution.

### *2.7.3 Washes and chemiluminescent detection*

The membranes were washed twice for 20 min at 68°C with 50 ml of prewarmed wash solution 1 (2 x SSC / 1% SDS), and twice for 20 min at 68°C with 50 ml of prewarmed wash solution 2 (0.1 x SSC / 0.5% SDS). They were then blocked for 40 min at room temperature with GEMatrix blocking solution and incubated for 40 min,



shaking, with alkaline-phosphatase-conjugated streptavidin diluted 1:5000 in GEAblocking solution. The membranes were then washed 3 times for 5 min in 10 ml of 1 x washing buffer F and rinsed twice in 1 x alkaline phosphatase detection buffer G before incubation for 5 min with CDP-star<sup>TM</sup> (Roche) chemiluminescent substrate. The image was captured by a 15 min exposure to film, as the Kodak Image Station 440cf imaging system was not sensitive enough to detect chemiluminescent signals from the array. To compare expression in two samples, the membranes were exposed simultaneously.

## 2.8 MOUSE MAST CELL PROTEASE-1 (mMCP-1) ELISA

### 2.8.1 Method 1

mMCP-1 in mouse serum was measured using a previously described ELISA (Scudamore *et al*, 1997). Standards were diluted from a stock solutions of 20 ng/ml mMCP-1 in PBS / 4% BSA / 0.05% Tween<sub>20</sub> with 2% mouse serum carrier protein. Dilutions of standards, samples and antibodies were in PBS / 4% BSA / 0.05% Tween<sub>20</sub>. ELISA plates (Immulon, Dynex technologies, Billingham, U.K.) were coated overnight at 4°C with 50 µl of affinity-purified rat anti-mMCP-1 monoclonal antibody (RF6.1) diluted to 5 µg/ml in carbonate buffer, pH 9.6. They were then washed 6 times in 0.9% NaCl / 0.05% Tween<sub>20</sub>, and incubated for 1 h at 37°C with standards and unknown samples (50 µl) before a further 6 washes and incubation for 30 min with 50 µl of sheep anti-mMCP-1 (2 µg/ml (Huntley *et al*, 1990)). Plates were then washed again and incubated for 30 min with 50 µl of donkey anti-sheep IgG-peroxidase (Sigma) conjugate diluted 1:8000, before a further 6 washes and a 20

min incubation with 50 µl of TMB substrate. The reaction was stopped with 50 µl of 0.18 M H<sub>2</sub>SO<sub>4</sub>, and the optical density of the reaction products read at 450 nm.

### 2.8.2 Method 2

This method was the result of optimisation of the previous method 1 in an attempt to reduce background. The ELISA protocol was as in method 2.8.1, with the following exceptions:

1. The affinity-purified rat anti-mMCP-1 monoclonal antibody RF6.1 was coated at a dilution of 2 µg/ml.
2. Bound mMCP-1 was detected by incubation with 50 µl of biotinylated sheep anti-mMCP-1 diluted at 1:1000 in PBS / 4% BSA / 0.05% Tween<sub>20</sub> followed by 6 washes with 0.9% NaCl / 0.05% Tween<sub>20</sub> and incubation for 30 min with 50 µl of streptavidin biotinylated horseradish peroxidase complex diluted at 1:4000 in PBS / 4% BSA / 0.05% Tween<sub>20</sub>.

## 2.9 ELECTROPHORESIS AND WESTERN BLOTTING WITH IMMUNODETECTION

### 2.9.1 Sample preparation

Stripped epithelial samples or mBMMC (1 x 10<sup>7</sup> mBMMC/ml of buffer) were vortexed vigorously in 2% SDS with protease inhibitors (Complete™ mini EDTA-free protease inhibitor cocktail tablets, Roche), and passed repeatedly through a 23 gauge needle to rupture the cells. They were then centrifuged for 15 min at 10000g and supernatant stored at -70°C. Whole jejunal samples were freeze-thawed once

and homogenised on ice in high salt buffer (PBS / 0.5 M NaCl / 0.05% Tween<sub>80</sub>).

They were then centrifuged for 15 min at 10000g and supernatants stored at -70°C.

## 2.9.2 SDS-PAGE

### 2.9.2.1 *Standard Laemmli gel system*

Gels were made up as in table 2.8. The protein content of the extracts was estimated by spectrophotometry (absorbance at 280 nm) to ensure similar loading. 75 µl of each extract was diluted 50:50 in sample buffer (table 2.9) and heated to 100°C for 5 min before loading onto a 6 cm long well. 5 µl of broad-range rainbow markers (Amersham, Little Chalfont, Buckinghamshire, U.K.) were diluted and heated similarly, and run on each gel. Gels were run at 200 V for 45 min in 0.3% Tris / 1.44% glycine / 0.1% SDS running buffer.

### 2.9.2.2 *Schägger gel system and detection of chemokines*

Due to the low molecular weight of the proteins, chemokines from jejunal epithelial and jejunal protein extracts were separated using the Schägger gel system. The protein content of extracts was measured using a BCA protein assay kit (Pierce, Rockford, USA) and 25 µl of jejunal extract (approximately 2 mg/ml total protein) or jejunal epithelial extract (approximately 1 mg/ml total protein) were loaded onto the gel; this was the maximum protein loading possible. Gels were made up as in table 2.10 and samples were diluted 50:50 in tricine sample buffer (table 2.11) and heated to 100°C for 5 min before loading onto the gel. 5 µl of low-range markers (Bio-Rad, Hemel Hempstead, U.K.) were similarly heated and run on each gel. Gels were run

Solution	Running gel		Stacking gel
	10%	12%	
H <sub>2</sub> O (ml)	4.05	3.35	6.1
1.5 M Tris-HCl, pH 8.8 (ml)	2.5	2.5	2.5
10% SDS (μl)	100	100	100
30% acrylamide / 0.8% bis-acrylamide (ml)	3.3	2.5	1.3
10% ammonium persulphate (μl)	100	100	100
TEMED (μl)	10	10	10

**Table 2.8. Gel solutions, standard Laemmli gel system.**

H <sub>2</sub> O	4.0
0.5 M Tris-HCl, pH 6.8	1.0
Glycerol	0.8
10% SDS	1.6
β-mercaptoethanol	0.4
0.05% (w/v) bromophenol blue	0.2
Final vol.	8.0

**Table 2.9. Sample buffer, standard Laemmli gel system (vol. in ml).**  
62.5 mM Tris-HCl, pH 6.8 / 10% glycerol / 2% SDS / 5% β-mercaptoethanol.

Gel type -	Stacking	Spacer	Resolving
Distilled H <sub>2</sub> O (ml)	2.48	3.2	-
3 M Tris 0.3% SDS pH 8.45 (ml)	0.96	3.3	3.3
Glycerol (g)	-	-	1.25
30% acrylamide / 0.8% bis-acrylamide (ml)	0.53	3.3	5.5
10% ammonium persulphate (μl)	50	100	100
TEMED (μl)	10	10	10
Total volume (ml)	4	10	10

**Table 2.10. Gel solutions, Schagger gel system.**

4 X 0.5 M TrisCl / 0.4% SDS pH 6.8 (ml)	2.4
Glycerol (ml)	2.4
SDS (g)	0.8
Dithiothreitol (g)	0.31
Coomassie blue G-250 (mg)	2
H <sub>2</sub> O (ml)	To 10 ml
Final vol (ml)	10

**Table 2.11. Tris-tricine sample buffer.**

at 50 V until the protein had entered the resolving gel, when the voltage was increased to 100 V for approximately 2 h or until the dye front was within 1 cm of the bottom of the gel. In a separate experiment (fig 2.8), gels were stained with Coomassie blue or silver staining to confirm presence of proteins in the chemokine molecular weight range (6 - 20 kDa) (2.9.3 and 2.9.4).

### **2.9.3 Staining of gels and membranes**

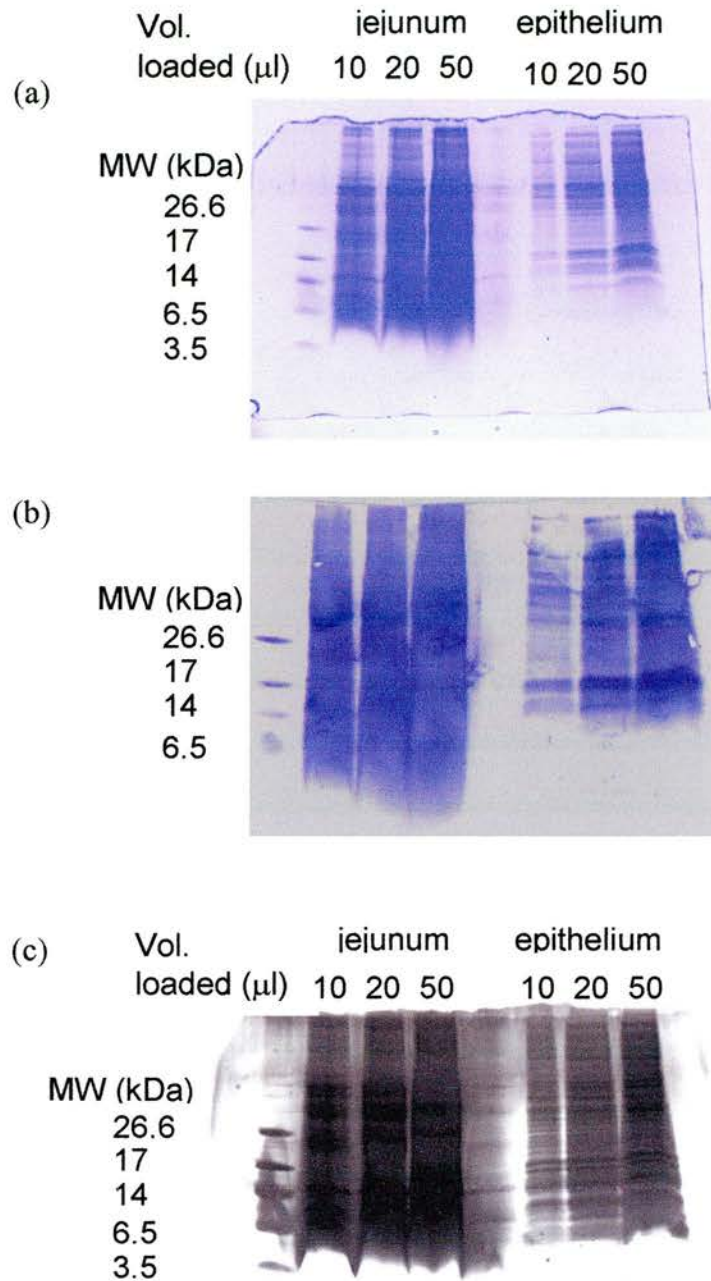
#### **2.9.3.1 Coomassie blue**

Gels were fixed and stained for 15 min in 0.5% Coomassie brilliant blue (R250, Sigma) dissolved in 25% methanol / 10% acetic acid. They were then destained in 50% methanol / 10% acetic acid for 1 - 2 h with 2 - 3 changes of destain.

To check transfer of protein, membranes were stained for 5 min in 0.5% Coomassie brilliant blue, as above, and destained briefly in 100% methanol followed by 1 h in 50% methanol / 10% acetic acid with 2 - 3 changes of destain (figure 2.8)

#### **2.9.3.2 Silver stain**

Gels were fixed for 15 min in 50% methanol / 10% acetic acid. They were then washed twice for 5 min in 5% methanol / 7% acetic acid and incubated for 15 min in dithiothreitol (5 µg/ml) in distilled water before washing 3 times for 5 min in distilled water. Gels were then incubated for 20 min in 0.1% silver nitrate, rinsed for 30 s in distilled water and developed using 100 ml 3% Na<sub>2</sub>CO<sub>3</sub> with 50 µl of 40% formaldehyde. Development was stopped by the addition of citric acid crystals.



**Figure 2.8. Separation of jejunal and epithelial extracts on a Schagger gel.**

- (a) Coomassie blue-stained gel.
- (b) Coomassie blue-stained immobilon-P membrane showing transfer of proteins.
- (c) Gel stained using silver stain (2.9.3.2) in order to show, more clearly, presence of proteins in the chemokine molecular weight range (6 - 20 kDa).

#### *2.9.4 Western blotting*

Proteins were transferred to polyvinylidene difluoride (Immobilon-P, Millipore) using the semi-dry method of blotting (Kyhse-Andersen, 1984). The transfer buffer was 10 mM 3-[Cyclohexylamino]-propane-sulphonic acid (CAPS, Sigma) in 10% methanol, and transfer was achieved in 1 h with a current of 80 mA / gel. Membranes were stained with Coomassie to check transfer, as in 2.9.3.1.

#### *2.9.5 Immunodetection of chemokines*

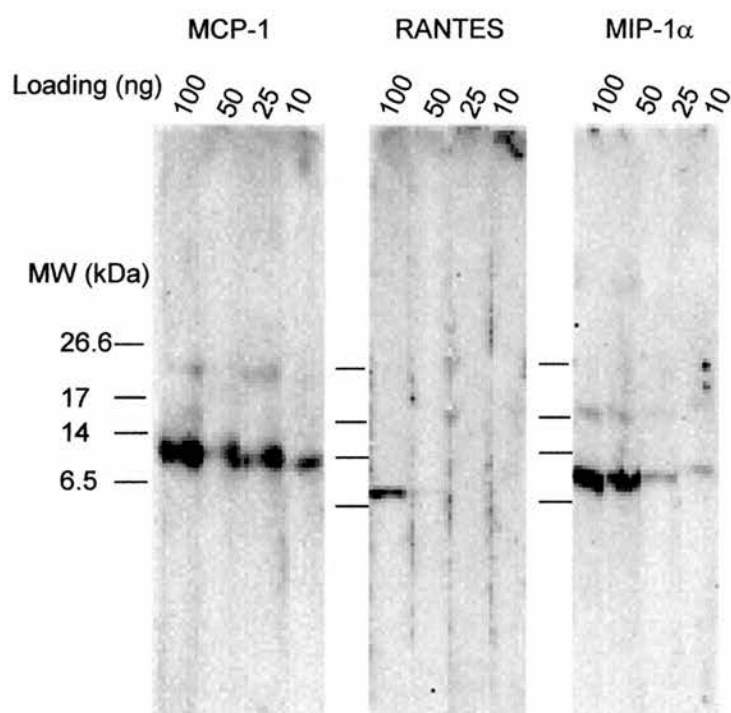
Preliminary studies (not shown) established optimum antibody concentrations and block buffer for this protocol. Membranes were blocked overnight at 4°C in PBS / 1% Marvel / 0.05% Tween<sub>20</sub> (block buffer). They were then incubated for 1 h with primary antibodies (Santa Cruz Biotechnology goat polyclonal anti MCP-1, RANTES or MIP-1 $\alpha$  antibodies (Autogen Bioclear, Calne, Wiltshire, U.K.)) at a dilution of 1:100 in block buffer before being washed 3 times in PBS / 0.05% Tween<sub>20</sub> and incubated for 30 min with rabbit anti-goat-alkaline phosphatase secondary antibodies (Jackson ImmunoResearch, Stratattech, Luton, U.K.) diluted 1:1000 - 1:10000 in block buffer. Membranes were then washed a further 3 times, rinsed briefly in detection buffer, and signals detected using CDP-star<sup>TM</sup> chemiluminescent substrate (Roche).

#### *2.9.6 Detection of chemokine protein standards using Santa Cruz*

##### *Biotechnology goat polyclonal antibodies*

Antibodies were tested using recombinant murine chemokine protein standards to show that chemokines could be recognised under the conditions used. 100, 50, 25

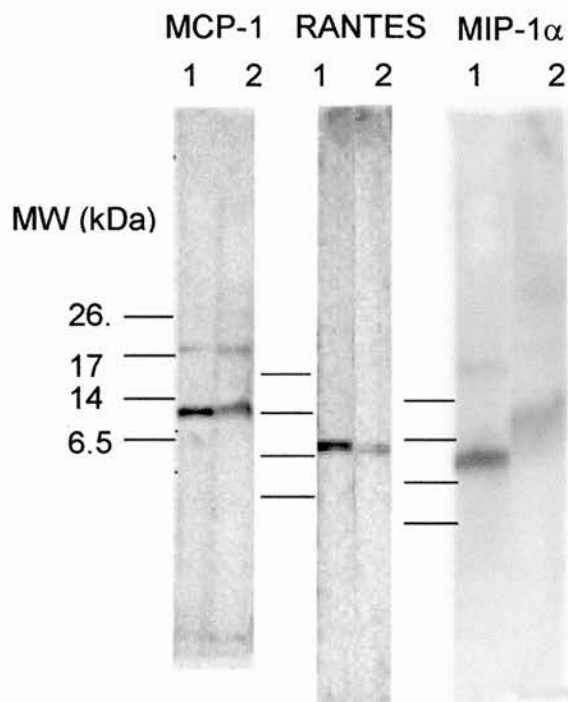




**Figure 2.9. Western blots of different loadings of chemokine standards.**

Murine recombinant chemokine protein standards were electrophoresed on a Schagger gel, transferred to immobilon-P and detected using Santa Cruz Biotechnology goat anti-chemokine polyclonal antibodies at 1:100 with rabbit anti-goat-AP secondary antibody at 1:10000.

10 ng of MCP-1 and MIP-1α were detectable, but RANTES was easily detectable only when 100 ng was loaded.



**Figure 2.10. Western blots of chemokine standards ± jejunal homogenates.**

10 ng of MCP-1 and MIP-1α and 100 ng RANTES were electrophoresed on a Schagger gel alone (1) or added to 25 µl of jejunal extract (2).

Proteins were transferred to Immobilon-P and detected using Santa Cruz Biotechnology goat anti-chemokine polyclonal antibodies at 1:100 with rabbit anti-goat-AP secondary antibody at 1:10000.

MCP-1 and RANTES run in the same place when added to jejunal extract, MIP-1α is running at a higher MW in this example.

and 10 ng of MCP-1, RANTES and MIP-1 $\alpha$  were electrophoresed, blotted and detected as described above using primary antibodies at a dilution of 1:100 and secondary antibody at 1:10000. 10 ng of MIP-1 $\alpha$  and MCP-1 were easily detectable (figure 2.9), but 50 ng was the limit of detection for RANTES. 10 ng of MCP-1 or MIP-1 $\alpha$  and 100 ng of RANTES were used as positive controls in subsequent experiments. Additionally, the above amounts of protein "spiked" into jejunal extracts were also shown to be detectable using this method (figure 2.10), although MIP-1 $\alpha$  ran at a higher MW in jejunal extracts in some experiments.

#### *2.9.7 Immunodetection of laminin binding protein (LBP)*

Membranes were blocked overnight at 4°C in TBS / 1% Marvel / 0.05% Tween<sub>20</sub> (block buffer) and cut into 0.5 cm strips, which were incubated for 1 h with primary antibody at a dilution of 1:100 in block buffer, or block buffer alone. Primary antibody was the rabbit anti-LBP polyclonal serum HK 149, which was raised to a 17-mer synthetic peptide from the NH<sub>2</sub>-terminal of murine LBP (residues 25 - 41), and was kindly provided by Dr H. Kleinmann, National Institute of Dental Research, NIH, Bethesda, U.S.A. The control antibody was normal rabbit serum (Sigma). Membranes were then washed 3 times in TBS / 0.05% Tween<sub>20</sub>, and incubated for 30 min with 2° antibody (mouse anti-rabbit IgG alkaline phosphatase conjugate, Sigma) diluted 1:20000 in block buffer. Membranes were washed a further 3 times and rinsed briefly in TBS before detection using 5-bromo-4-chloro-3-indoyl phosphate / nitroblue tetrazolium (BCIP/NBT) (Sigma-fast tablets).

## 2.10 IMMUNOHISTOCHEMISTRY

### *2.10.1 Antigen retrieval in paraformaldehyde-fixed sections*

Slides were dewaxed in xylene and hydrated through graded alcohols then covered in 10 mM Sodium Citrate buffer, pH 6 in a "Tender Cooker" microwave pressure cooker (Biogenics, CA, U.S.A.) and heated in a microwave until the buffer was boiling. Sections were then simmered for 4 min, and allowed to cool for 20 min prior to immunostaining as detailed below.

### *2.10.2 Preparation of frozen sections*

Proximal jejunum from male BALB/c mice was opened longitudinally and wrapped around the barrel of a plastic pipette in a "swiss roll" style with the luminal surface outward. The samples were then immersed in OCT compound (BDH), immobilised on cork discs and snap frozen in isopentane cooled on dry ice. Samples were stored at -70°C until ready for use, when 6 µm sections were cut using a cryostat (Shandon). Sections were stored at -70°C, and were thawed and allowed to air-dry overnight at room temperature before fixation for 10 min in acetone at -20°C and rinsing twice in PBS before use.

### *2.10.3 Immunohistochemical detection of mMCP-1 expression in cytospin preparations of mBMMC*

Paraformaldehyde - fixed slides (method 2.4.9) were rinsed in deionised water before blocking for endogenous peroxidase in 1% v/v hydrogen peroxide in methanol for 20 min. They were then rinsed for 5 min in tap water, blocked for 30 min in PBS / 0.5 M NaCl / 0.5% Tween<sub>80</sub> (blocking buffer) and incubated for 1 h with the anti-

mMCP-1 monoclonal RF6.1 (Scudamore *et al*, 1997) supernatant diluted 1:10 in block buffer or, for control purposes, with 1 µg/ml rat IgG1 (BD Pharmingen). Slides were then washed twice in PBS and incubated for 30 min with biotinylated anti-rat IgG (Vector laboratories, Peterborough, U.K.) diluted 1:100 in block buffer before being washed twice more and incubated for 30 min with Vectastain ABC *Elite* avidin-biotin detection system, made up according to the manufacturer's instructions (Vector labs). They were then incubated for 5 min with DAB peroxidase substrate (Vector labs) made up according to the manufacturer's instructions, washed twice in PBS, and counterstained for 5 min in 0.1% Mayers' haematoxylin solution (Sigma). Finally, sections were "blued up" in Scott's tap water before rinsing in tap water, dehydrating through graded alcohols, clearing in Xylene and mounting in DPX (Surgipath, Peterborough, U.K.)

#### *2.10.4 Immunohistochemical detection of chemokine expression in murine jejunum using Santa Cruz Biotechnology polyclonal antibodies*

After antigen retrieval of formalin fixed paraffin embedded sections, or fixation of frozen sections in acetone, sections were blocked for endogenous peroxidase activity by incubating for 20 min with 3% hydrogen peroxide in deionised water. However, due to difficulties in blocking endoperoxidase activity without loss of morphology in frozen sections using this method, endoperoxidase activity in frozen sections was blocked by a 10 min incubation with 100 µl of Dako peroxidase blocking reagent (Dako Ltd., Ely, U.K.). Sections were then incubated for 1 h in blocking solution (PBS / 1.5% rabbit serum) and washed twice in PBS before incubating with goat anti-chemokine polyclonal antibodies (Santa Cruz Biotechnology)(200 µg/ml) or normal goat IgG diluted 1:50 - 1:500 in blocking solution. These dilutions were as

suggested by the manufacturer's protocols. Sections were again washed twice in PBS and incubated for 30 min in biotinylated rabbit anti-goat IgG 2° antibody (Vector labs) at a dilution of 1:100 - 1:1000 before washing twice in PBS. Biotinylated antibody was detected using the ABC-DAB method, and sections were counter-stained, dehydrated and mounted (2.10.3).

#### *2.10.5 Immunohistochemical detection of MCP-1 using an R&D Systems polyclonal antibody*

The recommended R&D Systems method included paraformaldehyde fixation and permeabilisation using saponin. Thawed frozen sections were fixed for 10 min in 2% paraformaldehyde in PBS, pH 7.4. before rinsing in PBS and blocking endogenous peroxidase activity by incubating for 10 min with Dako peroxidase blocking reagent. Sections were then washed in PBS and blocked for 30 min in 1.5% rabbit serum / 0.01% saponin in PBS before incubating for 1 h with R&D Systems goat anti-mouse MCP-1 polyclonal antibody (100 µg/ml) or normal goat IgG diluted 1:40 or 1:80. Sections were then washed in PBS and incubated with biotinylated rabbit anti-goat 2° antibody (Vector labs) at 1:500 in 1.5% rabbit serum / 0.01% saponin in PBS before washing and detection of biotinylated antibody using the ABC-DAB method. Sections were then counter-stained, dehydrated and mounted (2.10.3).

## 2.11 ADHESION ASSAYS

### 2.11.1 Coating of plates

All coating proteins were obtained from Sigma and solubilised and stored as recommended (appendix 1, table A1.1). Proteins were diluted to the required concentration in sterile PBS and 96 well ELISA plates (Immulon) were coated overnight at 4°C with 100 µl of diluted protein. Excess proteins were then removed, and non-specific binding blocked by incubation for 2 h at 37°C with 3% BSA in PBS.

### 2.11.2 Adhesion assay

mBMMC or MC/9 cells were washed twice in PBS / 0.1% BSA before resuspending at  $5 \times 10^5$  cells/ml in their respective basic culture media (without cytokines or T-stim, including FCS). 100 µl of cells were then loaded in quadruplicate into wells and incubated for 1 or 2 h at 37 °C / 5% CO<sub>2</sub>, after which non-adherent cells were aspirated, and the wells washed 3 times with PBS. The number of cells adherent to wells was estimated using the β-hexoseaminidase assay (Landegren, 1983). Standard wells were prepared by the addition of the required volumes of cells at  $5 \times 10^5$  /ml to make  $0.25 - 5 \times 10^4$  cells/well, and the volume made up to 100 µl with PBS. When adhesion of two mast cell types was compared, separate standards were made up for each so that differences in β-hexoseaminidase content would not affect estimations of adhesion. Test wells were also made up to 100 µl volumes with PBS and plates were incubated at 37°C for 2 h with 60 µl of substrate buffer (7.5 mM p-nitrophenol -N-β-glucosaminide, Sigma). The reaction was stopped by the addition of 90 µl of

stop buffer (50 mM glycine / 5 mM EDTA, pH 10.5), and the optical density of the reaction products was read at 405 nm.

### *2.11.3 Stimulation of mBMMC adhesion by FcεRI cross-linking or treatment with calcium ionophore A23187*

For stimulation by FcεRI cross-linking, mBMMC were first sensitised by incubation overnight in complete culture medium with 100 ng/ml of IgE anti-DNP (Sigma). They were then washed twice in PBS / 0.1% BSA and resuspended in culture medium (without cytokines) before loading into wells and immediate addition of 10 ng/ml of DNP-HSA (Sigma). This protocol was based on unpublished work in our laboratory investigating stimulation of degranulation of mBMMC<sup>T+</sup> by FcεRI cross-linking.

For stimulation by calcium ionophore, mBMMC, washed and resuspended in culture medium without cytokines, were loaded into wells and 1 μM calcium ionophore A23187 (Sigma) immediately added. Cells to which 50 ng/ml of PMA (Sigma) was added immediately after loading into wells were included as a positive control for stimulation of adhesion and, as a negative control, cells to which no activating agent was added were included. In some experiments, additional controls of IgE-sensitised cells to which no antigen was added, and unsensitised cells to which antigen was added were included. The adhesion assay was then performed as in 2.11.2.



## 2.12 ANTIBODIES USED IN FLOW CYTOMETRY AND BLOCKING STUDIES

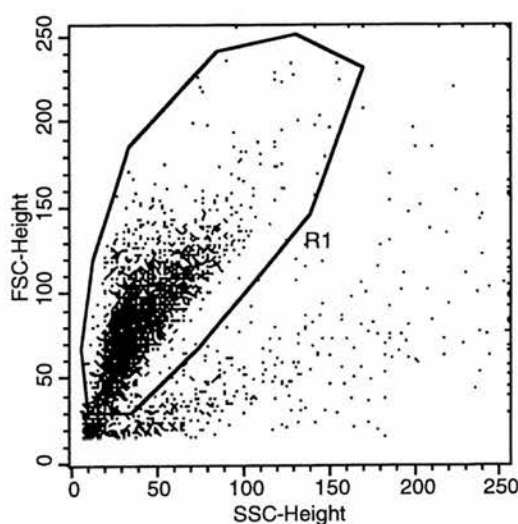
Mouse IgG1 monoclonal antibodies to  $\alpha 7\beta 1$  integrins were kindly provided by Dr H. von der Mark (Institute of Experimental Medicine, Friedrich-Alexander-University Erlangen-Nurnberg, Erlangen, Germany). They were obtained by immunisation of  $\alpha 7$ -deficient mice with wild-type primary myoblasts, as described (Schöber *et al*, 2000). Clone 6A11 was used for inhibition of adhesion of mBMMC<sup>T+</sup> to laminin-1 since it has the strongest adhesion-inhibition activity of all the anti- $\alpha 7$  clones obtained. Clone 3C12 did not inhibit cell adhesion to laminin and was used for flow cytometry.

Murine IgG1 and rat IgG1 and IgG2a isotype controls, rat anti-mouse  $\alpha v$  IgG1 (clone RMV-7), rat anti-mouse  $\alpha 5$  IgG2a (clone 5H10-27), rat anti-mouse  $\alpha 6$  IgG2a (clone GoH3) and rat anti-mouse Fc $\gamma$ RIII/II (Fc block, clone 2.4G2) were obtained from BD Pharmingen. Biotinylated-anti rat IgG2a or IgG1 secondary antibodies were also obtained from BD Pharmingen and anti-mouse IgG1-Alexa Fluor 488 was obtained from Molecular Probes (Leiden, The Netherlands).

## 2.13 FLOW CYTOMETRY

Surface expression of integrins was analysed by flow cytometry, with linear amplification for forward/side scatter and logarithmic amplification for FITC green fluorescence or phycoerythrin red fluorescence. This was performed by Mr. Andrew Sanderson, Institute of Cell, Animal and Population Biology, Department of Biological Sciences, University of Edinburgh using a FACScan (Becton Dickinson).

The machine was set for the population of cells to be analysed by ensuring that forward scatter, side scatter and fluorescence fell within the analytical range of the instrument. Events were gated to exclude erythrocytes and dead cells on the basis of forward and side scatter (R1, figure 2.11). Each population of cells tested comprised  $10^5 - 10^6$  cells and each flow cytometric analysis was based on  $10^4$  cells. Results were displayed as histograms with log fluorescence intensity along the x-axis (arbitrary units), and number of cells on the y-axis.



**Figure 2.11. Flow cytometric analysis of integrin expression in mBMMC.**

Example of a gate (R1) used to exclude erythrocytes and dead cells from the analysis.

### *2.13.1 Flow cytometric analysis of expression of $\alpha 5$ , $\alpha 6$ and $\alpha v$ integrins by mBMMC using rat monoclonal antibodies*

$1 \times 10^6$  mBMMC cells were incubated for 5 min with  $0.5 \mu\text{g}$  of murine Fc block followed by incubation for 1 h with  $1 \mu\text{g}$  of rat monoclonal antibody or isotype control in PBS 10% mouse serum. The cells were then washed twice in wash buffer (WB)(PBS / 0.1% BSA) and incubated for 30 min with  $1 \mu\text{g}$  of biotin-anti rat IgG2a

or IgG1 in PBS 10% mouse serum before washing twice and incubating for 30 min with 100  $\mu$ g of streptavidin-phycoerythrin (Vector labs). After labelling, cells were washed twice and fixed for 10 min in 2% paraformaldehyde before analysis by flow cytometry; all procedures were performed on ice.

#### *2.13.2 Flow cytometric analysis of $\alpha$ 7 integrin expression by mBMMC using the mouse anti-mouse $\alpha$ 7 monoclonal antibody, 3C12*

Optimum conditions for this experiment were established by pilot experiments incubating 3C12 or isotype control at 1, 2, 4 or 10  $\mu$ g/  $10^6$  cells; and secondary antibody at 1:2000 or 1:5000.

$2 \times 10^5$  cells were incubated for 5 min with 0.1  $\mu$ g of murine Fc block before incubation for 1 h with 2  $\mu$ g of the mouse anti- $\alpha$ 7 3C12, or mouse IgG<sub>1</sub> control in PBS 5% FCS. They were then washed twice in wash buffer and incubated for 30 min with anti-mouse IgG1-Alexa Fluor 488 diluted 1:2000 in PBS 5% FCS. After labelling, cells were washed and fixed as above. All procedures were performed on ice.

### **2.14 OPTIMISATION OF MC/9 CELLS AS POSITIVE CONTROLS FOR INVESTIGATION OF THE ROLE OF $\alpha$ 6 INTEGRIN IN ADHESION OF mBMMC<sup>T+</sup> TO LAMININ-1**

#### *2.14.1 Adhesion of MC/9 cells to ECM proteins*

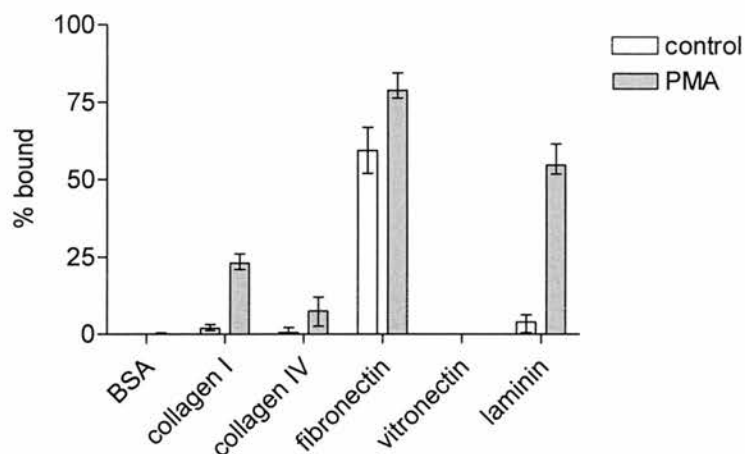
MC/9 cells are reported to adhere to laminin-1, and this adhesion can be blocked by the  $\alpha$ 6-neutralising antibody, GoH3. Therefore, these cells were cultured for use as a

positive control in experiments investigating the effect of GoH3 on adhesion of mBMMC<sup>T+</sup> to laminin-1 (6.5). An initial experiment was set up to confirm that MC/9 cells would adhere to laminin-1 using the adhesion assay developed to investigate adhesion of mBMMC to ECM proteins. In addition, adhesion of MC/9 cells to type I collagen, type IV collagen, fibronectin and vitronectin was analysed, as described (2.11.1 - 2.11.2). Proteins were coated at 20 µg/ml, with the exception of vitronectin, which was coated at 10 µg/ml; adhesion to wells coated with 3% BSA in PBS was also measured as a negative control. Adhesion of unstimulated cells and cells stimulated with 50 ng/ml PMA, which was added immediately after loading of cells into wells, was analysed after incubation for 1 h.

MC/9 cells adhered spontaneously to fibronectin and, after treatment with PMA, they also adhered to laminin-1 and type IV collagen (figure 2.12). Adhesion to laminin-1 confirmed their potential usefulness as a positive control for use of GoH3.

#### *2.14.2 The effect of preincubation of MC/9 cells with GoH3 on adhesion to laminin-1*

The above experiment (2.14.1) showed that MC/9 cells adhered to laminin-1 (20 µg/ml) following stimulation with PMA, but in order to establish optimum conditions for blocking experiments, the effect of preincubation with the anti-α6 integrin monoclonal, GoH3 at 1, 5 or 10 µg/ml on adhesion to laminin-1 was investigated. MC/9 cells were preincubated with GoH3 or IgG2a isotype control for 5 min at room temperature, as preliminary experiments (2.15.1) showed these to be

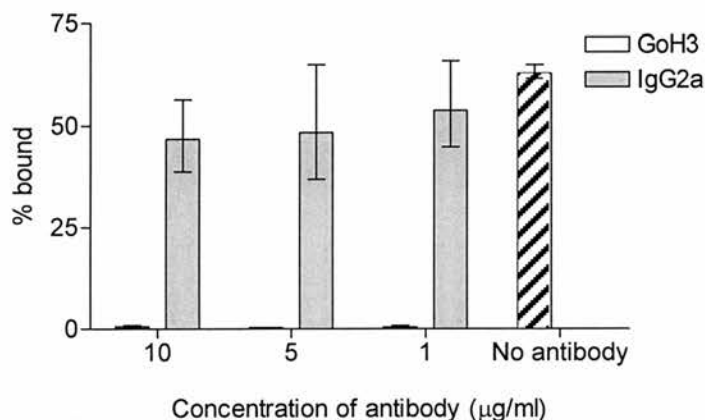


**Figure 2.12. Adhesion of MC/9 cells to extracellular matrix proteins.**

Each protein was coated at 20  $\mu\text{g/ml}$  except vitronectin, which was coated at 10  $\mu\text{g/ml}$ . 3% BSA is included as a negative control.

MC/9 cells adhered spontaneously to fibronectin and, following stimulation with PMA, there was increased adhesion to fibronectin, laminin-1 and collagens.

Results are median (range) of one experiment,  $n=4$  wells.



**Figure 2.13. Antibody blocking of the adhesion of MC/9 cells to laminin-1.**

MC/9 cells were preincubated for 5 min at room temperature with 1, 5 or 10  $\mu\text{g/ml}$  of the anti- $\alpha 6$  integrin monoclonal GoH3 or IgG2a isotype control.

Preincubation with GoH3 at all concentrations examined blocked adhesion of MC/9 cells to laminin-1. Results are expressed as median (range) of one experiment,  $n=3$  wells.

optimum conditions. Adhesion to laminin-1 after incubation for 1 h was then analysed as described (2.11.2). GoH3 ( $\geq 1 \mu\text{g/ml}$ ) blocked adhesion of MC/9 cells to laminin-1, whereas preincubation with isotype control IgG2a ( $\geq 1 \mu\text{g/ml}$ ) resulted in only a small and variable reduction in adhesion to laminin-1 (figure 2.13).

Inhibition of adhesion of MC/9 cells to fibronectin by preincubation with anti- $\alpha 5$  integrin antibody has been reported (Fehlner-Gardiner *et al*, 1996b), therefore preliminary experiments investigated use of these cells as a positive control for investigation of the role of  $\alpha 5$  in adhesion of mBMMC<sup>T</sup> to fibronectin. However, adhesion of MC/9 cells to fibronectin was not blocked by the  $\alpha 5$  neutralising monoclonal 5H10-27 (not shown), though flow cytometric analysis using the same antibody showed surface expression of this integrin. The reason for this was unknown, but these cells were not considered suitable as a positive control for blocking experiments using 5H10-27.

## 2.15 PREINCUBATION CONDITIONS FOR INVESTIGATION OF THE ROLE OF INTEGRINS IN ADHESION OF mBMMC TO ECM PROTEINS

### 2.15.1 Pilot experiments to optimise preincubation conditions

Conditions for preincubation of neutralising antibodies were established in pilot experiments (data not shown). Antibodies were preincubated at 1, 5 or 10  $\mu\text{g/ml}$  with mBMMC or MC/9 cells (2.14.2) for 5 - 30 min on-ice or at room temperature. These starting conditions were chosen on the basis of published work (Fehlner-

Gardiner *et al*, 1996a; Schöber *et al*, 2000), or manufacturers' data sheets for commercial antibodies. In all experiments, cells incubated with isotype control and with no antibody were included as negative controls.

#### 2.15.2 Preincubation of mBMMC<sup>T+</sup> with 6A11

The role of  $\alpha 7$  integrin in adhesion of mBMMC<sup>T+</sup> to laminin-1 was investigated by preincubation of mBMMC<sup>T+</sup> for 30 min on ice with the anti- $\alpha 7$  monoclonal 6A11 (10  $\mu$ g/ml). This concentration had previously been shown to inhibit adhesion of  $\alpha 7$ -transfected HEK293 cells to laminin-1 (Schöber *et al*, 2000).

#### 2.15.3 Preincubation of mBMMC<sup>T+</sup> with GoH3

Preincubation with GoH3 at up to 10  $\mu$ g/ml did not block adhesion of mBMMC<sup>T+</sup> to laminin-1 in preliminary experiments (not shown). Therefore MC/9 cells, adhesion of which to laminin-1 can be blocked by GoH3 (Fehlner-Gardiner *et al*, 1996a), were used as positive controls for this antibody. Optimum conditions were established using MC/9 cells (2.14.2), and MC/9 cells preincubated with GoH3 under these conditions were included in each experiment to investigate the effect of this antibody on adhesion of mBMMC<sup>T+</sup> to laminin-1.

To determine the role of  $\alpha 6$  integrin in adhesion of mBMMC<sup>T+</sup> to laminin-1, mBMMC<sup>T+</sup> or MC/9 cells were preincubated for 10 min at room temperature with the anti- $\alpha 6$  monoclonal GoH3 or isotype control (1  $\mu$ g/ml).

#### *2.15.4 Preincubation of mBMMC<sup>T-</sup> with 5H10-27 or RMV-7*

The role of  $\alpha 5$  in adhesion of mBMMC<sup>T-</sup> to fibronectin was investigated by preincubation for 10 min at room temperature with 1, 5 or 10  $\mu\text{g/ml}$  of 5H10-27 or isotype control in one pilot experiment, and with 10  $\mu\text{g/ml}$  in subsequent experiments. The role of  $\alpha v$  in adhesion of mBMMC<sup>T-</sup> to vitronectin was investigated by preincubation for 10 min at room temperature with 10  $\mu\text{g/ml}$  of RMV-7 or isotype control.

#### **2.16 STATISTICAL ANALYSIS**

Statistical analysis was performed using non-parametric tests with GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California, U.S.A. The Mann Whitney-U test was used to compare up to three groups of data ( $n=4$ ). Four or more groups of data ( $n=4$ ) were compared using the Kruskal-Wallis test with Dunn's multiple comparison post-test. Data are represented as graphs of median (range); when repeat experiments were performed; results are shown in tabular form in appendix 2.



## CHAPTER 3

### EXPRESSION OF MAST CELL CHEMOATTRACTANTS IN MURINE JEJUNAL EPITHELIUM FOLLOWING *N. BRASILIENSIS* INFECTION

#### 3.1 INTRODUCTION

Mast cell precursors are recruited into the jejunal mucosa following nematode infection (Kasugai *et al*, 1995), where they are thought to differentiate into mucosal mast cells (MMC) under the influence of cytokines expressed in the lamina propria, and subsequently migrate intraepithelially. The mechanisms directing this migration are not yet clear but, as described in 1.10, variations in chemokine expression regulate migration of other immune cells throughout the body, and mast cells are likely to be no exception to this regulatory system. In support of this, chemokines including MCP-1, MIP-1 $\alpha$ , RANTES, (Taub *et al*, 1995) and fractalkine (Papadopoulos *et al*, 2000) are chemotactic for several mast cell phenotypes.

There is considerable evidence that modulation of epithelial chemokine expression plays an important role in initiation of the immune response to intestinal infections by attracting the necessary spectrum of immune cells. Interaction of invasive bacteria stimulates upregulation of monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) in cultured colon epithelial cells (Jung *et al*, 1995) and rotavirus infection of cultured intestinal epithelium stimulates upregulation of macrophage inflammatory protein-1 $\beta$  (MIP-1 $\beta$ ), MCP-1 and RANTES (Rollo *et al*, 1999). Also, stimulation of epithelium by pro-inflammatory cytokines such as interleukin -1 (IL-

1) and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) can upregulate chemokine expression (Panja *et al*, 1995).

Less is known about the intestinal epithelial response to gut-dwelling parasites, but studies using both cell culture (Li *et al*, 1998) and epithelial isolation methods (Stadnyk & Kearsley, 1996) have shown increases in IL-1 $\beta$ , IL-8 and epithelial-neutrophil activating peptide-78 (ENA-78), which are chemoattractants for neutrophils, in response to infection with the nematode parasite, *Trichinella spiralis*.

Expression of mast cell chemoattractants by intestinal epithelium following gastrointestinal nematode infection has not been studied, and alterations in expression could attract mast cells or their precursors into the epithelium. Therefore, expression of the chemokines RANTES, MCP-1 and MIP-1 $\alpha$ , and cytokines SCF and TGF- $\beta_1$ , which are also mast cell chemoattractants (Meininger *et al*, 1992; Gruber *et al*, 1994), was investigated in a *N. brasiliensis* model of intestinal mastocytosis. In addition, expression of the chemokine TECK, which may attract IEL into the epithelium (Kunkel *et al*, 2000), was also investigated, since IEL and MMC may share common recruitment mechanisms. Epithelium from infected and uninfected mice was collected using a modification of the perfusion technique first described by Bjerknes and Cheng (Bjerknes & Cheng, 1981), thus enabling characterisation of the epithelial immune response to an *in vivo* infection.

## 3.2 RT-PCR ANALYSIS OF CHEMOKINE AND CYTOKINE EXPRESSION IN MURINE JEJUNAL EPITHELIUM FOLLOWING *N. BRASILIENSIS* INFECTION

### 3.2.1 *Experimental aim and design*

The aim of this experiment was to investigate the expression of putative mast cell chemoattractants and the lymphocyte chemoattractant, TECK, by jejunal epithelium of *N. brasiliensis* infected mice. Fifteen 12-week-old mice were infected with 500 *N. brasiliensis* larvae (2.2.1). After subcutaneous injection, *N. brasiliensis* migrates via the lungs arriving in the intestine between 48 and 72 h after infection. Numbers of MMC begin to increase on day 7 post-infection (Wastling *et al*, 1997), but in order to examine both early and late responses, mice were killed and expression of chemokines and cytokines in jejunal epithelium was examined (in triplicate) on days 1, 2, 4, 7 and 14 post-infection. Uninfected control mice were also included.

Jejunal epithelium was collected by the modified Bjerknes EDTA perfusion technique (2.3.2). Prior to euthanasia, anaesthetised mice were bled (2.3.1) and serum retained for measurement of mMCP-1 (2.8.1) to confirm that there was a mastocytosis in association with a successful infection (Wastling *et al*, 1997). Epithelium was collected into TRI reagent for RT-PCR, and into 4% paraformaldehyde for morphological examination (2.3.3); and after perfusion, a 2 cm section of whole jejunum was collected into RNA *later*<sup>TM</sup> and processed for use in RT-PCR (2.3.4).

Expression of transcripts for MCP-1, MIP-1 $\alpha$ , RANTES, TECK, fractalkine, TGF- $\beta_1$ , SCF and mMCP-1 in epithelial RNA samples was detected by RT-PCR (2.5.1 - 2.5.7 and table 2.1). As very low expression of MCP-1 was detected in epithelial samples, expression of this chemokine in whole jejunum was also measured by RT-PCR. Also, initial experiments suggested increased MCP-1 expression in jejunal epithelium on day 7 post-infection, therefore this was further investigated using semi-quantitative PCR (2.5.8).

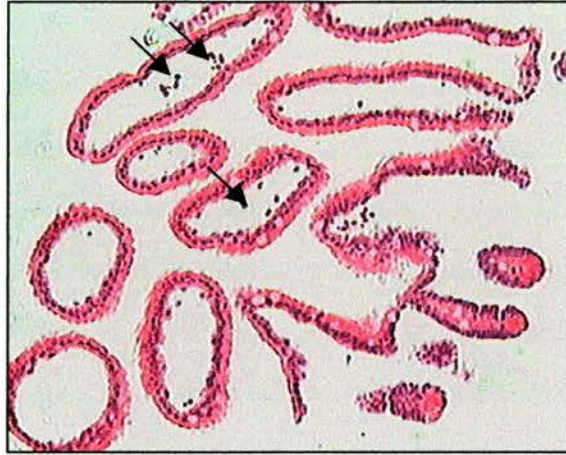
### 3.2.2 *Results*

#### 3.2.2.1 *Examination of stripped epithelium*

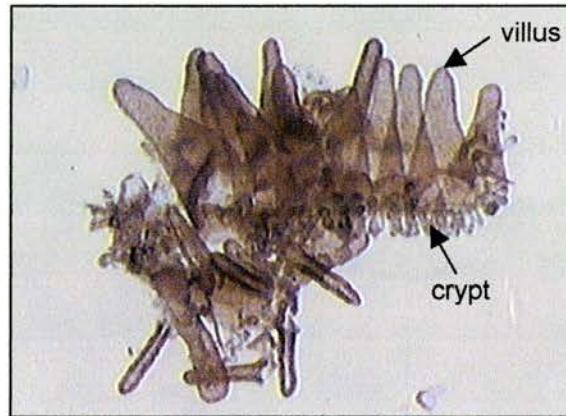
Examination of stripped epithelium under low power using a dissecting microscope showed that collected samples consisted of epithelium in small sheets, discrete crypt-villous units or separated crypts and villi (figure 3.1). Haematoxylin and eosin-stained histological sections confirmed this and, with the exception of one sample, levels of contaminating non-epithelial cells were below 5% (table 3.1). Differential expression of MCP-1 in whole jejunal and epithelial samples (see below) further supported evidence that these represented two different cell populations.

The Bjerknes method of epithelial isolation is superior to other chelating, enzymic or mechanical methods because intact pieces of epithelium are recovered with little contamination with lamina propria cells and with high cell viability (Bjerknes & Cheng, 1981). Our modification of this method was easier to perform and resulted in a similar recovery of epithelium, with comparable viability (>95%).

(a)



(b)



**Figure 3.1. Exfoliated jejunal epithelium obtained by the modified Bjerknes technique.**

(a) Haematoxylin and eosin stained epithelial preparations (x 100) showing the relative absence of non-epithelial elements. A few contaminating non-epithelial cells are present (arrows)(see table 3.1).

(b) Whole mount of freshly isolated epithelium viewed with the dissecting microscope (x 25). Note that both villi and crypts are recovered by this method.

Time post-infection (days)	n=	% contaminating cells
0	3	3.6 (2.0 - 7.6)
1	3	2.4 (2.2 - 2.4)
2	2	3.0 (3.0 - 3.0)
4	3	2.2 (1.8 - 3.6)
7	3	3.0 (1.8 - 4.6)
14	2	1.8 (1.6 - 2.0)

**Table 3.1. Contamination of epithelial preparations with other cell types.**

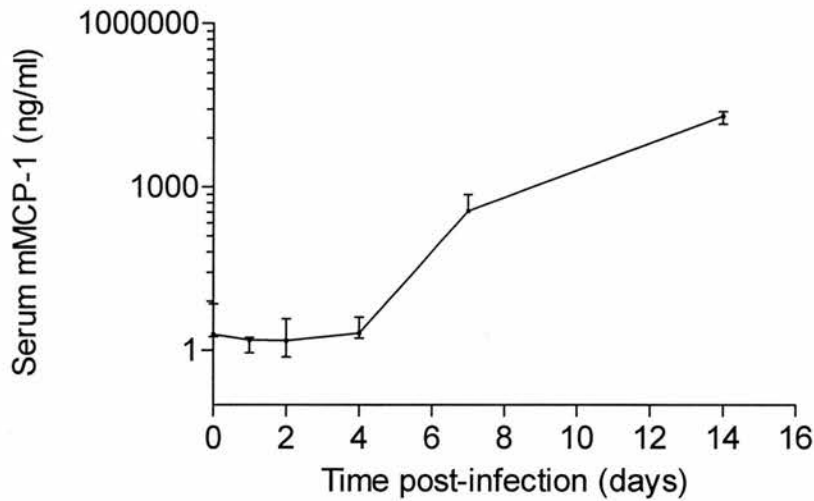
500 epithelial cells were counted in haematoxylin and eosin stained preparations, and the number of associated non-epithelial cells estimated. Contaminating cells consisted of mainly lymphocytes with some neutrophils.

Results are expressed as median (range); n= number of preparations examined.

#### 3.2.2.2 Expression of mMCP-1 in serum and epithelium

Serum mMCP-1 levels and levels of mMCP-1 mRNA in jejunal epithelium were measured as an indication of mast cell numbers in the jejunum. The mast cell response to *N. brasiliensis* has been previously characterised, and the increase in mast cell numbers shown to be closely paralleled by increased serum and jejunal mMCP-1 (Scudamore *et al*, 1997; Wastling *et al*, 1997).

mMCP-1 was detected in the serum of all mice, but was at very low levels prior to day 7. Median (range) serum mMCP-1 concentrations on day 4 post-infection were 4.0 ng/ml (1.7 - 4.1 ng/ml) (n=3 mice). Serum mMCP-1 concentrations were increased above control values on days 7 (0.4 µg/ml (0.07 - 0.7 µg/ml)) and 14 (20 µg/ml (0.014 - 24.4 µg/ml)) post-infection (figure 3.2).



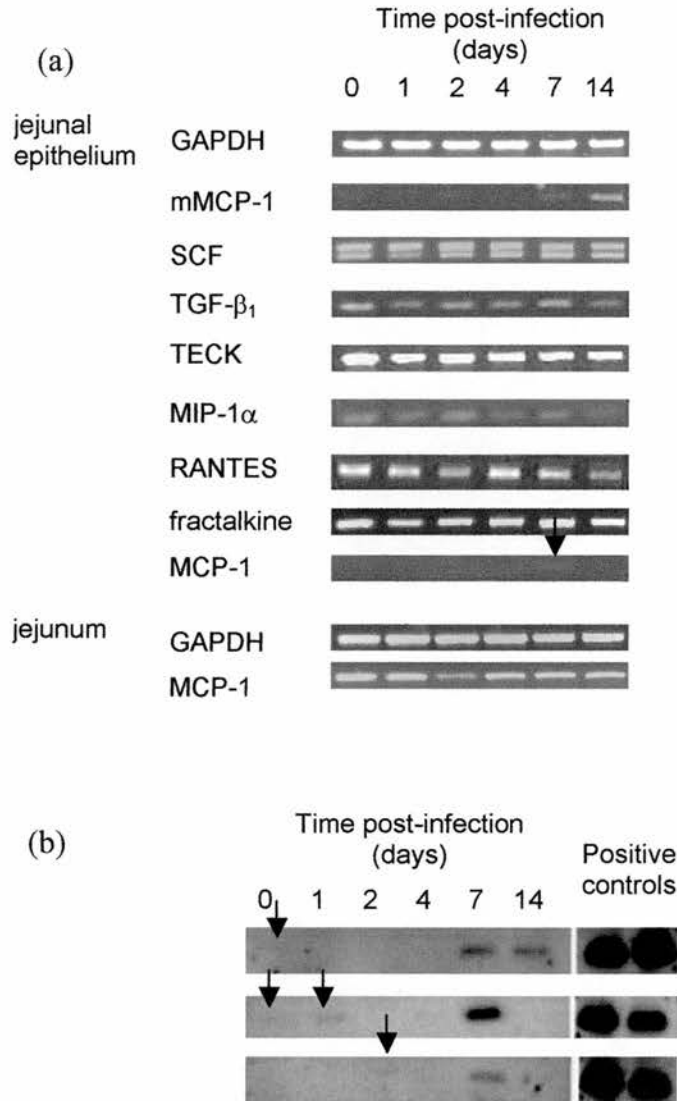
**Figure 3.2. Serum mMCP-1 concentrations in male BALB/c mice following infection with *N. brasiliensis*.**

Values are median (range) (n=3 mice).

To confirm the intraepithelial location of MMC, expression of mMCP-1 was measured in jejunal epithelial samples by RT-PCR. Expression was detected in all samples collected on days 7 and 14 post-infection (figure 3.3), but low-to-undetectable levels of expression were seen in samples on days 0 (uninfected controls), 1 and 2 and 4 post-infection. This is consistent with a previous study of jejunal mMCP-1 expression using Northern blotting (Wastling *et al*, 1997).

### 3.2.2.3 Expression of chemokines and cytokines in jejunal epithelium

Expression of mRNA for the chemokines MIP-1 $\alpha$ , RANTES, TECK and fractalkine, and of the cytokines SCF and TGF- $\beta_1$  was detected in both control (uninfected) epithelial samples and in epithelial samples collected at all time-points from infected



**Figure 3.3. Expression of cytokines and chemokines in murine jejunal epithelium or jejunum during infection with *N. brasiliensis*.**

a) RT-PCR - Expression of mMCP-1 on days 7 and 14 confirms movement of mast cells into the epithelium as a result of infection. All chemoattractants are constitutively expressed with the exception of MCP-1, which is detected only on day 7 (shown by arrow) in this example. For comparison, jejunal MCP-1 expression is shown. It is constitutive and expression appears to be at much higher levels than in epithelium.

Results are representative of data from 3 separate mice. Upper and lower bands of the SCF signal represent transmembrane and secreted forms, respectively.

b) Southern blot of PCR products from amplification of jejunal epithelial cDNA using primers specific for MCP-1. Arrows indicate faint bands. Positive controls are whole jejunal samples, in which there is strong expression of MCP-1.

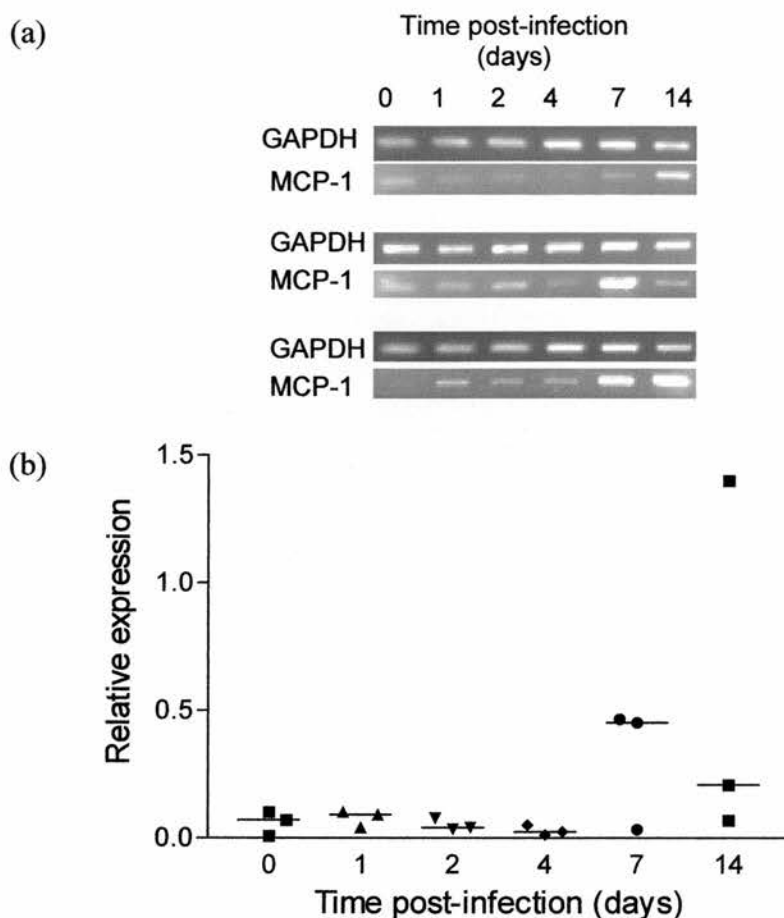


mice (figure 3.3). No consistent changes in expression were detected using this method. All PCR products were of the expected size, and hybridised, as expected, to complementary oligonucleotide probes (table 2.1).

Expression of the chemokine MCP-1 was initially undetectable by RT-PCR in any sample except those collected on day 7, and one sample collected on day 14 post-infection (figure 3.3). Southern blotting revealed low levels of expression in 2 control samples, 2 samples collected on day 1 and in one sample collected on day 2 post-infection, and confirmed the increased levels of expression in all day 7 samples and one day 14 sample (figure 3.3). Use of 10-fold more cDNA for semi-quantitative PCR showed low level expression of MCP-1 in all samples, with increased expression in 2 samples collected on day 7 and 2 samples on day 14 post-infection (figure 3.4).

#### *3.2.2.4 Expression of MCP-1 in whole jejunum*

In contrast to that seen in epithelium, MCP-1 expression was detected by RT-PCR in control jejunal samples, and at all time points post-infection (figure 3.3.) No increase in expression was detected following *N. brasiliensis* infection. Expression of MCP-1 in jejunum was more abundant than in epithelium (figure 3.3).



**Figure 3.4. Expression of MCP-1 in jejunal epithelium during infection with *N. brasiliensis*, analysed using semi-quantitative RT-PCR.**

(a) Ethidium bromide-stained gels showing MCP-1 and respective GAPDH signals.

(b) Scatter graph of semi-quantitative MCP-1 expression in intestinal epithelium.

Values plotted are ratios of intensity of MCP-1 signal / GAPDH signal for 3 sets of animals.

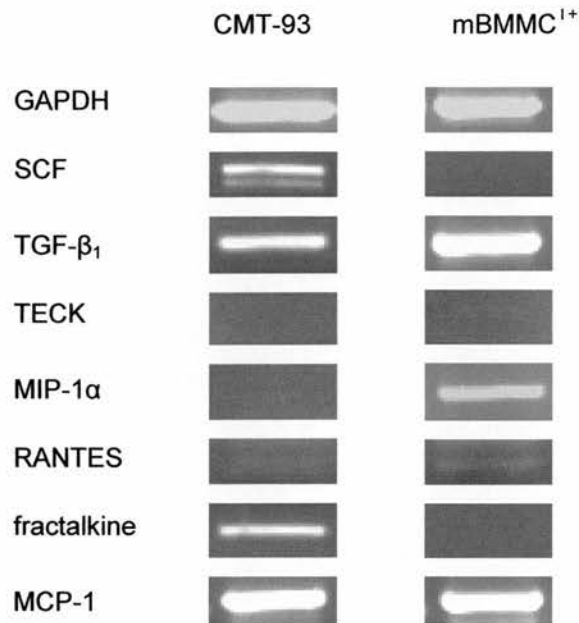
Points represent relative expression in epithelium from individual mice and horizontal bars indicate medians.

### 3.3 EXPRESSION OF CHEMOKINES AND CYTOKINES BY CULTURED MAST CELLS AND THE CMT-93 MURINE INTESTINAL EPITHELIAL CELL LINE

#### 3.3.1 *Experimental aim and design*

The previous experiment showed expression of several cytokines and chemokines from intestinal epithelium, and upregulation of MCP-1 expression following infection with *N. brasiliensis*. Several cell types within epithelium may express these molecules including epithelial cells, IEL and mast cells. The aim of the following experiment, therefore, was to investigate the likely source of expression of the cytokines and chemokines detected in 3.2.

Expression of transcripts for MCP-1, MIP-1 $\alpha$ , RANTES, TECK, fractalkine, TGF- $\beta_1$  and SCF in the epithelial cell line CMT-93, which was derived from a murine rectal carcinoma, and in cultured mBMMC<sup>T+</sup> (MMC homologues) was investigated. CMT-93 cells and mBMMC<sup>T+</sup> were cultured (2.4.1 and 2.4.3), and cells collected for RNA extraction (2.4.10.) Expression of cytokines and chemokines was analysed in 3 cultures of both mBMMC<sup>T+</sup> and CMT-93 cells by RT-PCR (2.5.1 – 7); specific conditions for each primer were as in 3.2, and are detailed in table 2.1.



**Figure 3.5. Expression of chemoattractants by cultured mBMMC<sup>T+</sup> and the murine intestinal epithelial cell line, CMT-93.**

SCF, TGF- $\beta_1$ , fractalkine and MCP-1 were expressed in CMT-93 cells; whereas expression of TGF- $\beta_1$ , MIP-1 $\alpha$  and MCP-1 was detected in mBMMC<sup>T+</sup>.

RANTES was also expressed at low levels by mBMMC<sup>T+</sup> and inconsistently by CMT-93 cells. Expression of TECK was detected inconsistently and at very low levels in both cell types.

### 3.3.2 Results

RT-PCR analysis showed expression of a number of cytokines and chemokines by mBMMC<sup>T+</sup> and CMT-93 epithelial cells. Both MCP-1 and TGF- $\beta_1$  were strongly expressed by both mBMMC<sup>T+</sup> and CMT-93 epithelial cells (figure 3.5). Expression of MIP-1 $\alpha$  was detected in mBMMC<sup>T+</sup> but not in CMT-93 epithelial cells. In contrast, expression of fractalkine and SCF was detected in CMT-93 epithelial cells, but not mBMMC<sup>T+</sup> (figure 3.5). RANTES was expressed at very low levels in

mBMMC<sup>T+</sup> and inconsistently in CMT-93 epithelial cells; TECK was detected inconsistently and at low levels in mBMMC<sup>T+</sup> and CMT-93 epithelial cells.

### 3.4 USE OF A MACROARRAY FOR ANALYSIS OF CHEMOKINE EXPRESSION IN MURINE JEJUNAL EPITHELIUM FOLLOWING *N.* *BRASILIENSIS* INFECTION

#### 3.4.1 *Experimental aim and design*

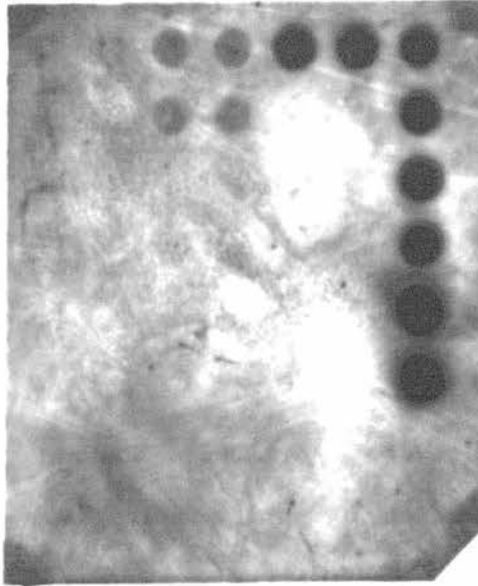
Constitutive expression of a number of cytokines and chemokines was detected in intestinal epithelium, but expression of the majority of these remained unchanged after infection with *N. brasiliensis* (3.2.2.3). Expression of MCP-1 was upregulated, but levels of this chemokine remained low, even in infected epithelium. It was thought possible, however, that expression of other chemokines not previously investigated may be altered following *N. brasiliensis* infection. A mouse chemokine Non-rad (non radioactive) GEMatrix™ macroarray kit (2.7) was therefore used to investigate expression of chemokines in jejunal epithelium collected from uninfected mice, and mice infected 7 days previously with *N. brasiliensis*.

Epithelium from three infected and three uninfected mice was collected into TRI reagent (2.3.3), and RNA extracted and DNase treated (2.5.1 - 2.5.2). Expression of chemokines was then examined in combined uninfected and infected epithelial samples using the GEMatrix kit, using separate membranes for uninfected and infected samples (2.7).

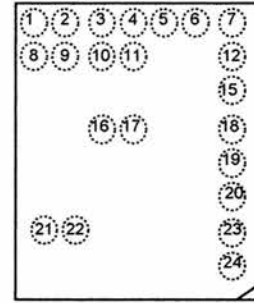
#### 3.4.2 *Results*

The chemokines analysed by the GEMatrix are shown in figure 2.7. Use of the GEMatrix™ to detect expression of chemokines in intestinal epithelium confirmed

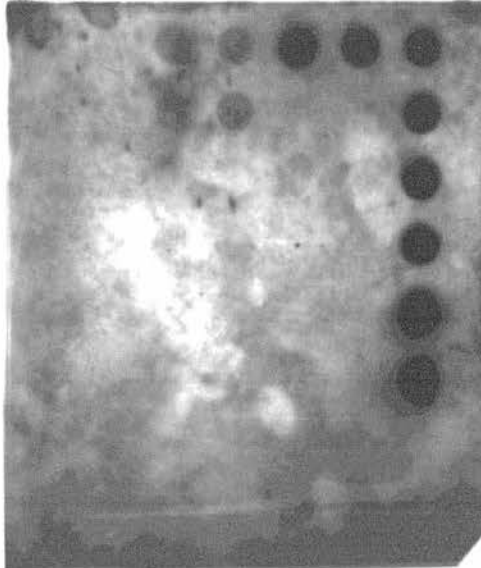
Control



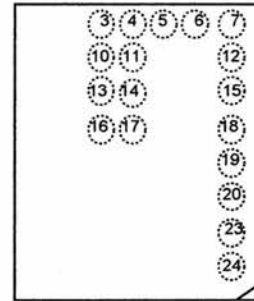
Key



Infected



Key



**Figure 3.6. Expression of chemokine transcripts in murine jejunal epithelium.**

Epithelium was collected from uninfected (control) mice and from mice infected 7 days previously with *N. brasiliensis*.

Chemokines TECK (3, 4), RANTES (10, 11) and MIP-1 $\alpha$  (16, 17) were clearly detected in both control and infected epithelium. Expression of SDF-2 (1, 2), PF-4 (8, 9) and fractalkine (21, 22) was less obvious in control epithelium, and MIP-2 (13, 14) may be expressed in infected epithelium.

5, 6, 7, 12, 13, 18 are GAPDH controls, 19 and 20 are  $\beta$ -actin controls, and 23 and 24 are bacterial plasmid negative controls.

several results from RT-PCR and revealed expression of 2 other chemokines in uninfected epithelium (figure 3.6). Strong expression of TECK and RANTES, and weaker expression of MIP-1 $\alpha$  in both uninfected and infected epithelium was confirmed (figure 3.6). However, expression of fractalkine was detected only in uninfected epithelium, but higher background in this area of the array may have prevented detection in the infected sample (figure 3.6). Weak expression of chemokines SDF-2 and PF4 was detected in uninfected, but not in infected epithelium (figure 3.6).

### 3.5 INVESTIGATION OF CHEMOKINE PROTEIN EXPRESSION IN MURINE JEJUNUM BY WESTERN BLOTTING

#### 3.5.1 *Experimental aim and design*

The experiments described in 3.3 and 3.4 established that transcripts encoding chemokines and cytokines are constitutively expressed in intestinal epithelium. It was of interest, therefore, to determine whether the relevant protein products could also be detected by Western blotting. In this preliminary study, expression of RANTES, MIP-1 $\alpha$  and MCP-1 protein was investigated in uninfected epithelial and whole jejunal samples since transcripts for RANTES and MIP-1 $\alpha$  in epithelium, and of MCP-1 in whole jejunum, were detected both in the presence and absence of infection.

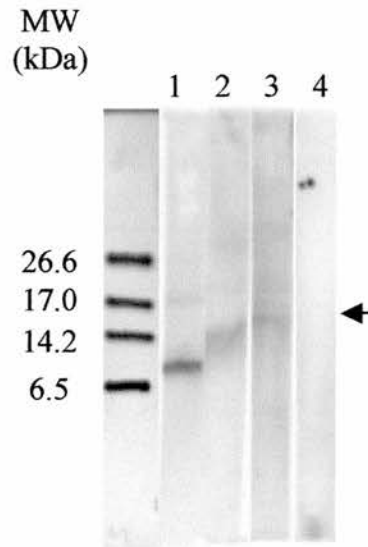
Protein was extracted (2.9.1) from previously collected and stored epithelial or whole jejunal samples (2.3.2 - 2.3.4). Samples were electrophoresed on a Schagger gel (2.9.2.2) and proteins transferred onto Immobilon inert supports (2.9.4). Chemokines

were detected using an immunodetection protocol with chemiluminescent detection (2.9.5). In each experiment, 10 ng (100 µg RANTES) of protein standards (recombinant murine chemokine proteins, Autogen Bioclear, Calne, Wiltshire, UK) and 10 ng (100 ng RANTES) of protein standards "spiked" into epithelial or whole jejunal extracts were also run alongside test samples as positive controls. These standards were derived through titration experiments (2.9.6).

### 3.5.2 Results

Investigation of epithelial expression of RANTES, MIP-1 $\alpha$  and MCP-1 by Western blotting produced variable results. Though chemokine standards were readily detected on blots (figures 2.9 and 2.10), no chemokines were detected in intestinal epithelium, and neither RANTES nor MCP-1 were detected in homogenates of whole jejunum (data not shown). Antibodies to RANTES appeared least sensitive, as 100 ng was the minimum amount of standard detected as opposed to 10 ng standards for MCP-1 and MIP-1 $\alpha$  (2.9.6, figure 2.9); and positive controls for RANTES were inconsistently detected, therefore results from experiments to detect RANTES were considered unreliable. A positive band was detected on blots in jejunal samples using polyclonal antibody to MIP-1 $\alpha$  (figure 3.7) in 3 separate experiments using 2 different jejunal extracts. This band was of higher molecular weight (15 kDa) than the expected 8 kDa, but MIP-1 $\alpha$  was detected as a 15 kDa band in blots from jejunal samples "spiked" with standard.





**Figure 3.7. Western blot to detect MIP-1 $\alpha$  in uninfected murine jejunum.**

Lane 1 - 10 ng murine recombinant MIP-1 $\alpha$  standard.

Lane 2 - jejunal extract + 10 ng MIP-1 $\alpha$  standard.

Lanes 3 and 4 - jejunal extract.

Lanes 1 - 3 were incubated with goat anti-MIP-1 $\alpha$  polyclonal primary antibody (1:100) and lane 4 (negative control) was incubated with normal goat polyclonal antibody (1:100).

Standards (lanes 1 and 2) were detected using rabbit anti-goat AP secondary antibody at a dilution of 1:10000, whereas incubation with secondary antibody at a dilution of 1:5000 was required for detection of MIP-1 $\alpha$  in jejunum only (lanes 3 and 4).

### 3.6 INVESTIGATION OF CHEMOKINE PROTEIN EXPRESSION IN MURINE JEJUNUM BY IMMUNOHISTOCHEMISTRY

#### 3.6.1 *Experimental aim and design*

As results from Western blotting to detect chemokines in jejunal samples were equivocal (3.5.2), immunohistochemistry was used in an attempt to show expression of chemokine protein in murine jejunum and in jejunal epithelium. Additionally, RT-PCR showed much higher expression of MCP-1 in whole jejunum than in epithelium, and we were interested to know the cellular source of this expression. The goat polyclonal antibodies (Santa Cruz Biotechnology) used for Western blotting were also used in immunohistochemical protocols. Expression of MIP-1 $\alpha$ , MCP-1 and RANTES was investigated by immunohistochemistry (2.10.4) in paraformaldehyde-fixed (2.4.9) sections following antigen retrieval (2.10.1). Jejunum from control mice, and from mice infected with *N. brasiliensis* 7 and 10 days earlier was used for these experiments. Expression was also investigated using acetone-fixed frozen sections (2.10.2) from an uninfected mouse by immunohistochemistry (2.10.4).

In addition to Santa Cruz antibodies, further attempts were made to detect MCP-1 in frozen sections from an uninfected mouse using a goat anti-MCP-1 polyclonal antibody obtained from R&D Systems. The method was as recommended by R&D Systems (2.10.5).

### 3.6.2 *Results*

Although Western blotting showed detection of recombinant chemokine standards (figure 2.9), no convincing staining for any of the chemokines was detected using Santa Cruz polyclonal antibodies or the R&D systems anti-MCP-1 polyclonal antibody in the fixed and frozen tissue sections examined (not shown).

### 3.7 DISCUSSION

Upregulation of several chemokines and cytokines occurs in epithelium in response to viruses (Rollo *et al*, 1999), bacteria (Jung *et al*, 1995; Eck *et al*, 2000) and nematode parasites (Stadnyk & Kearsley, 1996; Li *et al*, 1998). The present study showed constitutive expression of transcripts for a number of chemokines and cytokines in murine intestinal epithelium, and increased expression of MCP-1 following *N. brasiliensis* infection.

MCP-1 is well-established as a monocyte chemoattractant and is chemotactic for murine IL-3-dependent bone marrow mast cells (Taub *et al*, 1995) and mouse skin mast cells (Conti *et al*, 1997). Expression of MCP-1 has been correlated with an increase in mast cell numbers in wound healing and in chronic airway inflammation (de Boer *et al*, 2000; Trautmann *et al*, 2000). In the intestinal tract, MCP-1 expression has been detected by immunohistochemistry in normal colonic epithelium (Reinecker *et al*, 1995), lamina propria and endothelial cells (Mazzucchelli *et al*, 1996) making it a potential candidate for mast cell recruitment to the intestine. The high levels of expression of MCP-1 seen in whole jejunum in this study (3.2.2.4) suggest it could be involved in initial recruitment of mast cell precursors from the circulation into tissues, which normally occurs as early as day 3 post-infection of mice with *Strongyloides venesuelensis* (Tegoshi *et al*, 1997). Although the increased expression of MCP-1 identified in epithelium may be important in intraepithelial migration of MMC, it seems unlikely that a significant chemotactic gradient could be established against the apparently higher expression levels in the lamina propria.

The precise source of expression of MCP-1 and of the other molecules examined in the epithelial fraction is unknown, but may include both epithelial cells and intraepithelial inflammatory cells such as lymphocytes and mast cells. Cultured murine epithelial (CMT-93) cells strongly expressed MCP-1, TGF- $\beta_1$ , SCF and fractalkine and expressed low levels of RANTES and TECK (3.3.2). Expression of RANTES (Yang *et al*, 1997), TECK (Muehlhoefer *et al*, 2000) and fractalkine (Wurbel *et al*, 2000) has also been demonstrated previously in intestinal epithelial cells or epithelial cell lines.

Expression of MCP-1, (Baghestanian *et al*, 1997), MIP-1 $\alpha$  and RANTES (Selvan *et al*, 1994) has been reported in mast cells, and mBMMC<sup>T+</sup> cultured in our lab also expressed these three chemokines, and TGF- $\beta_1$  (3.3.2). Expression of these molecules by newly recruited intraepithelial mast cells could contribute to the apparent increase in MCP-1 against the low background expression of this chemokine in epithelium. Intraepithelial lymphocytes (IEL) are able to express MIP-1 $\alpha$ , RANTES and TGF- $\beta_1$  (Boismenu *et al*, 1996; Kearsey & Stadnyk, 1996) and may contribute to constitutive expression in exfoliated epithelium. Alterations in the population of IEL following infection were not examined in this study, however, other authors have shown a marked decrease in numbers of IEL following infection with the nematode parasite *Trichinella spiralis* (Garside *et al*, 1992; Bozic *et al*, 1998). If a similar decrease was seen following *N. brasiliensis* infection, this could make IEL a less likely source of the chemoattractants examined, although RANTES is a major product of IEL (Shires *et al*, 2001).

The chemokines MIP-1 $\alpha$  and RANTES, which are also chemotactic for mast cells, (Taub *et al*, 1995; Conti *et al*, 1998) were expressed constitutively in intestinal epithelium, but upregulation was not seen following *N. brasiliensis* infection. This was surprising since these molecules have been shown to be upregulated in other inflammatory diseases including asthma (Teran, 2000) and inflammatory bowel disease (Mazzucchelli *et al*, 1996; Vainer *et al*, 1998). Additionally, upregulation of RANTES expression in a rat model of colitis was correlated with an increase in mast cell numbers, this increase was abrogated by use of Met-RANTES, a specific antagonist of RANTES receptors CCR1 and CCR5 (Ajuebor *et al*, 2001).

The observation of constitutive expression of TECK and fractalkine is in agreement with previous studies, which have implicated these molecules in lymphocyte trafficking (Wurbel *et al*, 2000; Muehlhoefer *et al*, 2000), but fractalkine has recently also been shown to be chemotactic for mast cells (Papadopoulos *et al*, 2000). Expression may therefore contribute towards non-inflammatory recruitment of the small numbers of mast cells that reside in the non-parasitised jejunum.

Expression of the cytokines SCF and TGF- $\beta_1$  was also measured in epithelial samples since these molecules are both growth factors for MMC homologues (Miller *et al*, 1999), and chemoattractants for mast cells (Meininger *et al*, 1992; Gruber *et al*, 1994). Expression of SCF has been correlated with mast cell accumulation in arthritis (Ceponis *et al*, 1998) and pollen allergies (Nilsson *et al*, 1998); and is upregulated in intestinal epithelial cell cultures following infection with *Salmonella*

*typhimurium* (Klimpel *et al*, 1996). Both molecules were expressed in epithelium in this study, but no alteration in expression was seen following nematode infection. There was no evidence for a change in expression of the majority of the chemoattractants examined by RT-PCR following *N. brasiliensis* infection. One reason for this may be that invasion and damage of epithelial cells is necessary to evoke alteration in inflammatory gene transcription. This is considered to be the case for bacterial infection of the gut because of the high bacterial load of normal gut contents (Svanborg *et al*, 1999), and may explain the epithelial response to the invasive parasite, *T. spiralis* (Stadnyk & Kearsey, 1996; Li *et al*, 1998), though expression of a different set of chemokines was analysed in these studies. Alteration of chemokine expression in response to non-invasive parasites may, however, be possible via other mechanisms; excretory-secretory products are key allergens in the immune response to *N. brasiliensis* and those from plerocoids of the parasite *Spirometra erinaceieuropaei* can modulate chemokine expression in macrophages (Fukumoto *et al*, 1997). The limitations of RT-PCR in measurement of changes in gene expression must also be considered; small changes in expression may not be detected by the methods used and changes in expression may not correlate with protein production.

The specific recruitment of MMC following *N. brasiliensis* infection is difficult to explain in the light of this evidence of constitutive expression of several mast cell chemoattractants from intestinal epithelium, especially since chapter 4 shows that cultured mBMMC<sup>T+</sup> express several of the corresponding receptors for the chemoattractants expressed. However, mast cell recruitment may depend on other

chemoattractants that were not investigated in this study, or may be limited by other factors, such as seeding of mast cell precursors into tissues and expression of the necessary integrins or proteases to allow cells to move through the extracellular matrix.

Results from further investigation of the importance of other chemoattractants using a macroarray (3.4.2) were consistent with constitutive expression of RANTES, TECK and MIP-1 $\alpha$  in intestinal epithelium. The macroarray also showed low level expression of fractalkine, SDF-2 and PF-4 in uninfected epithelium. As PF-4 is chemotactic for mast cells (Taub *et al*, 1995), its expression may contribute towards mast cell recruitment. The function of SDF-2 has yet to be determined.

Because of the detection method used and the high background seen in the arrays, these results would be unsuitable for reliable comparison of expression between the two samples. Therefore, expression of chemokines only in uninfected epithelium would need to be confirmed by repeat experiments after refinement of the technique, and more quantitative techniques such as RT-PCR or real-time PCR.

Expression of other chemokines shown to be chemotactic for mast cells, including eotaxin (Romagnani *et al*, 1999; Ochi *et al*, 1999) and SDF-1 $\alpha$  (Juremalm *et al*, 2000), was also examined using the macroarray, but mRNA levels were probably below those detectable using this method.



The data from Western blots and immunohistochemistry were disappointing. It is possible that there were technical problems, but it is also likely that the techniques were not sensitive enough to detect low levels of protein. Only expression of MIP-1 $\alpha$  was suggested at protein level (3.5.2); the MW shift in the presence of jejunal extract may suggest dimer formation, as previously reported for MIP-1 $\alpha$  (Hoogewerf *et al*, 1997). Other chemokines may have been below levels detectable by Western blotting and immunohistochemistry using these antibodies.

Possibly because of low and transient expression, and the labile nature of these molecules, chemokines are difficult to detect in tissue sections. Consequently, a large number of studies rely on molecular techniques such as *in situ*-hybridisation (Mazzucchelli *et al*, 1996; Trautmann *et al*, 2000) and RT-PCR (Ajuebor *et al*, 2001). However, immunohistochemical detection of MCP-1 has been reported in human inflammatory bowel disease mucosa (Reinecker *et al*, 1995) and human COPD airways (de Boer *et al*, 2000), and expression of MIP-1 $\alpha$  was also detected by immunohistochemistry in ulcerative colitis mucosa, though inappropriate "no primary antibody" controls were used in this study (Vainer *et al*, 1998).

Reports of immunohistochemical detection of chemokines in mouse or rat tissue are few, possibly due to limited availability of suitable antibodies, but expression of MCP-1 has been detected in rat myocytes using a goat polyclonal antibody from Santa Cruz Biotechnology (Reyes-Reyna *et al*, 2002).

In conclusion, we have shown both increased expression of MCP-1 mRNA following *N. brasiliensis* infection, and constitutive expression of mRNA for the chemokines MIP-1 $\alpha$ , RANTES, TECK and fractalkine; and cytokines SCF and TGF- $\beta_1$  in murine intestinal epithelium. These molecules could contribute towards recruitment of mast cells or their precursors into the jejunal epithelium.

## CHAPTER 4

### CHEMOKINE RECEPTOR EXPRESSION DURING MAST CELL DIFFERENTIATION IN CULTURE

#### 4.1 INTRODUCTION

The experiments described in the previous chapter showed expression of a number of chemokines and cytokines by murine intestinal epithelium, and expression of putative mast cell chemoattractants MCP-1 (Mazzucchelli *et al*, 1996), RANTES (Reinecker *et al*, 1995), MIP-1 $\alpha$  (Vainer *et al*, 1998) and fractalkine (Muehlhoefer *et al*, 2000) has been shown in both normal and diseased human intestine. However, in order to be recruited into the mucosa and epithelium by these chemoattractants, MMC or their precursors would need to express the corresponding receptors.

Chemokines including MCP-1, MIP-1 $\alpha$ , RANTES, PF-4 (Taub *et al*, 1995), fractalkine (Papadopoulos *et al*, 2000), IL-8 (Lippert *et al*, 1998; Nilsson *et al*, 1999), SDF-1 $\alpha$  (Juremalm *et al*, 2000) and eotaxin (Romagnani *et al*, 1999; Ochi *et al*, 1999) are all chemotactic for mast cells *in vitro*, and expression of many of the corresponding chemokine receptors has been identified.

MMC, however, differ phenotypically from the cultured mast cells used for previous investigations of chemotaxis and chemokine receptor expression, and therefore may differ in their expression of chemokine receptors. A growing body of evidence suggests tissue-specific chemokine-mediated control of lymphocyte migration (Kunkel & Butcher, 2002), and similar mechanisms may apply to MMC. Therefore

expression of chemokine receptors by MMC may differ from that of mast cells in other tissues. As MMC are located intraepithelially, they may express chemokine receptors in common with IEL, which express CX3CR1, CCR3, CCR5 and CXCR3 (Muehlhoefer *et al*, 2000; Agace *et al*, 2000).

MMC differentiate from immature precursor cells during the course of their migration (Friend *et al*, 1996), and changes in chemokine receptor expression during mast cell differentiation may regulate this migration. Mast cell progenitors may express chemokine receptors important in initial recruitment across vascular endothelium, and those expressed by mature MMC could facilitate intraepithelial migration. Study of chemokine receptor expression by mast cell progenitors is limited by the difficulty of isolating these cells. However, in a recently developed *in vitro* culture system, murine bone marrow cells can be induced to differentiate into 100% MMC-like homologues after 10 days of culture with IL-3, IL-9, SCF and TGF- $\beta_1$  (Wright *et al*, 2002). Although this system does not enable examination of chemokine receptor expression by mast cell progenitors, investigation of chemokine receptor expression during differentiation of mBMMC<sup>T+</sup> would allow correlation with increasing numbers of mBMMC<sup>T+</sup> over time.

As a key regulator of the mucosal phenotype (Miller *et al*, 1999), TGF- $\beta_1$  may also regulate expression of chemokine receptors important in intraepithelial mast cell migration. Therefore, the initial aims of these experiments were to investigate chemokine receptor expression in mBMMC<sup>T+</sup>, to correlate trends in chemokine

receptor expression with differentiation of mBMMC<sup>T+</sup> *in vitro* and to investigate the role of TGF- $\beta_1$  in control of chemokine receptor expression in mBMMC.

## 4.2 PRELIMINARY INVESTIGATION OF CHEMOKINE RECEPTOR EXPRESSION DURING MAST CELL DIFFERENTIATION *IN VITRO* IN THE PRESENCE OR ABSENCE OF TGF- $\beta_1$

### 4.2.1 *Experimental aim and design*

Having described some of the chemokines present in normal and parasitised jejunum (chapter 3) the aim of this chapter was to investigate expression of chemokine receptors by mBMMC<sup>T+</sup> (MMC homologues). This preliminary experiment also aimed to investigate chemokine receptor expression during differentiation of mBMMC from bone marrow cells, and examine the effect of TGF- $\beta_1$  on chemokine receptor expression.

Bone marrow cells from 10 mice were pooled and allocated to mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> cultures for up to 14 days (2.4.1). On day 10, the mBMMC<sup>T-</sup> culture was divided into two and one culture supplemented with 10 ng/ml of anti-TGF- $\beta_1$  antibody, based on preliminary results from antibody titration (2.4.2), in order to abrogate the effect of endogenous TGF- $\beta_1$  on mast cell differentiation. At time-point 0 (at the time of bone marrow harvest), and on days 2, 4, 7 and 14 of culture,  $2.5 \times 10^6$  -  $1 \times 10^7$  mBMMC<sup>T+</sup> were removed into TRI reagent (2.4.10) for RNA extraction (2.5.1). mBMMC<sup>T-</sup> were removed only on days 7 and 14 of culture as at earlier time points, cells were used for titration of anti-TGF- $\beta_1$  antibody (2.4.2). Additionally, cytospin-preparations were made and stained with Leishman's (2.4.7), or fixed with paraformaldehyde (2.4.9) for mMCP-1 immunohistochemistry (2.10.3).

Expression of CCR1 - 5, and of CCR6 - 9 and CX3CR1 was examined in mBMMC using CytoXpress<sup>TM</sup> mPCR kits CCR set 1 and set 2, respectively. These sets include several chemokine receptors shown to be expressed by other mast cell phenotypes. Expression of CXCR2 and CXCR4 has also been shown in mast cells (Lippert *et al*, 1998; Nilsson *et al*, 1999; Juremalm *et al*, 2000) but primers amplifying these genes were not included in the mPCR kits, therefore expression was investigated using RT-PCR (2.5.1 – 2.5.7 and table 2.2).

#### 4.2.2 Results

Leishman-stained cytospin preparations from cell cultures confirmed that mouse bone marrow cells differentiated into mast cells, based on morphology (table 4.1). Quantification of mMCP-1 expression by immunohistochemistry (table 4.1) and RT-PCR (figure 4.1) confirmed that differentiation into MMC homologues occurred in cultures supplemented with TGF- $\beta_1$ .

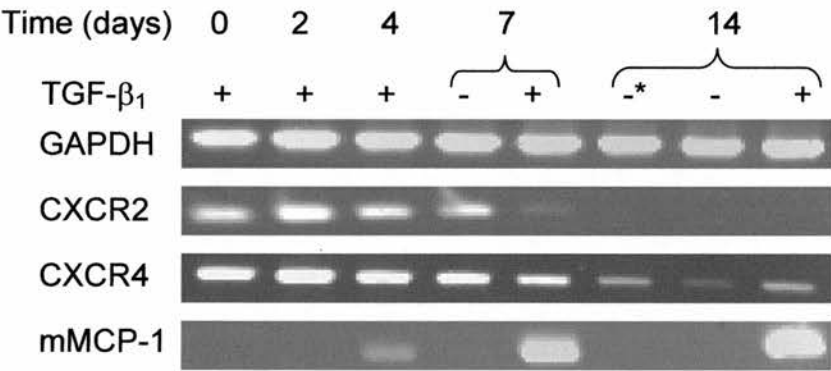
Culture	Time (days)	% MC (Leishman's)	% MMC <sub>h</sub> (mMCP-1 <sup>+</sup> )
mBMMC <sup>T+</sup>	0	0	0
	2	7	2
	4	53	29
	7	97	98
	14	99	100
mBMMC <sup>T-</sup>	7	81	6
	14	98	3
	14*	98	0

**Table 4.1. Cell counts from experiment 4.2.**

Results show numbers of mast cells (MC), as assessed by morphology on Leishman's staining, and mucosal mast cell homologues (MMC<sub>h</sub>), as assessed by immunohistochemical staining for expression of mMCP-1.

500 cells were counted, \* indicates inclusion of anti-TGF- $\beta_1$  antibody in this culture.

Expression of chemokine receptors varied during differentiation (figures 4.1 and 4.2), but there were no differences between mBMMC cultured with or without TGF- $\beta_1$ . Expression of CXCR2, CXCR4 (figure 4.1), CCR6 and CCR7 (figure 4.2b) appeared to decrease, whereas expression of CCR2 and CCR5 increased with time as the percentage of mast cells increased (figure 4.2a). CCR1 was expressed at all stages, low expression of CX3CR1 and CCR8 was detected in day 14 mBMMC<sup>T+</sup> and no expression of CCR3, 4 or 9 was detected in any cultures. On the basis of this, a larger scale experiment was set up to confirm trends in chemokine receptor expression during the differentiation of mBMMC<sup>T+</sup> in the presence of TGF- $\beta_1$ .



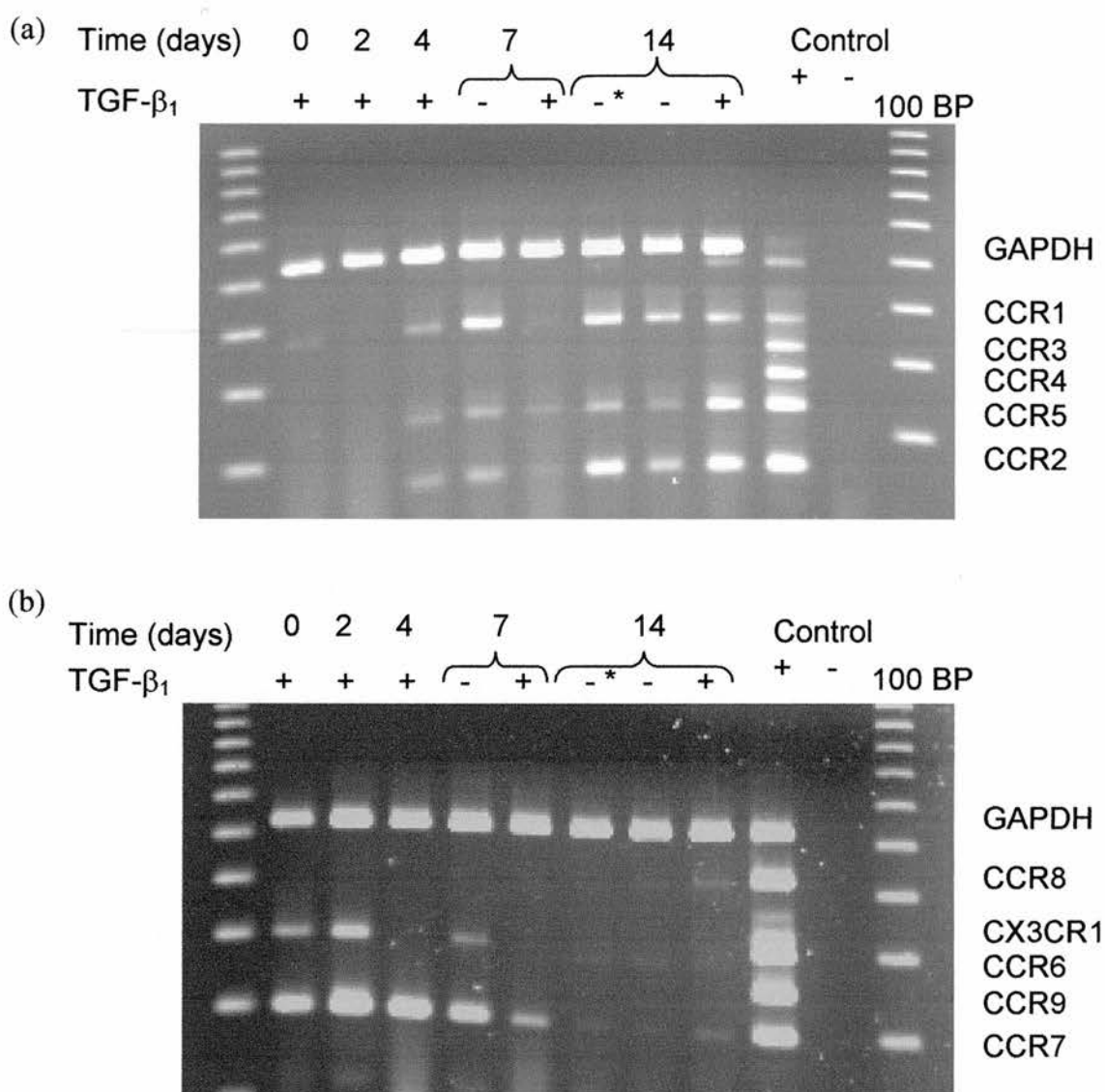
**Figure 4.1. Expression of chemokine receptors during mast cell differentiation in experiment 4.2.**

Downregulation of CXCR2 and CXCR4 expression occurred during mast cell differentiation. Expression of mMCP-1 indicated differentiation into MMC homologues in cultures supplemented with TGF- $\beta_1$ .

Expression of both CXCR2 and CXCR4 was comparable in day 14 mast cells cultured with and without the inclusion of TGF- $\beta_1$ .

\* indicates that this culture included chicken anti-human TGF- $\beta_1$  from day 10 onwards.





**Figure 4.2. Expression of chemokine receptors during mast cell differentiation in experiment 4.2.**

- (a) Analysis of CCR1 - 5 (CytoXpress™ CCR set 1 mPCR kit), showing expression of CCR1 in all samples and upregulation of expression of CCR2 and CCR5 during mast cell differentiation.
- (b) Analysis of CCR6 - 9 and CX3CR1 (CytoXpress™ CCR set 2 mPCR kit), showing high expression of CCR7 in early cultures, but low expression in day 14 mBMMC. Expression of CCR6 was also downregulated during mast cell differentiation. Expression of CCR8 and CX3CR1 was weakly detected in day 14 mBMMC.

Expression of chemokine receptors analysed using both multiplex kits was comparable in day 14 mast cells cultured with and without TGF- $\beta_1$ .

\* indicates that this culture included chicken anti-human TGF- $\beta_1$  from day 10 onwards.

### 4.3 CHEMOKINE RECEPTOR EXPRESSION DURING MAST CELL DIFFERENTIATION IN THE PRESENCE OF TGF- $\beta_1$

#### 4.3.1 *Experimental aim and design*

The aim of this experiment was to further explore and to provide statistical evaluation of the expression of chemokine receptors during mBMMC<sup>T+</sup> differentiation in the presence of TGF- $\beta_1$ . To ensure sufficient cell numbers on day 2 of culture, bone marrow cells from 7 mice were pooled for each of 4 replicate mBMMC<sup>T+</sup> cultures. Samples were collected and processed, and chemokine receptor expression investigated as in experiment 4.2, except that expression of CXCR2 and CXCR4 was analysed by semi-quantitative RT-PCR (2.5.8) and relative expression of chemokine receptors analysed using mPCR kits was also calculated as described (2.6.1). In this experiment, mast cell differentiation was monitored using both Leishman's (2.4.7) and toluidine blue (2.4.8) staining, as these are well-established methods for identification of mast cells (Enerbäck, 1966b).

#### 4.3.2 *Results*

Examination of Leishman-stained cells and enumeration of toluidine blue-stained cells in cytopsin preparations confirmed that cultured mouse bone marrow cells differentiated into mast cells (table 4.2). Differences between results from the two methods were largely due to difficulties in identification of immature cells by both methods in earlier cultures. 49% toluidine blue-positive cells on day 4 is similar to results reported previously (Wright *et al*, 2002), but proportions of mMCP-1<sup>+</sup> cells on day 4 and 7 were lower than in the previous experiment (table 4.1), and than previously reported (Wright *et al*, 2002). The reason for this was unknown, but may

have been the result of difficulties in accurately distinguishing positive cells against the high background staining during immunohistochemical detection of mMCP-1 for this experiment. However, as expected, 100% of mBMMC<sup>T+</sup> on day 14 expressed mMCP-1 (table 4.2) and RT-PCR analysis of expression of mMCP-1 (figure 4.3a) further confirmed the differentiation of bone marrow cells into MMC homologues.

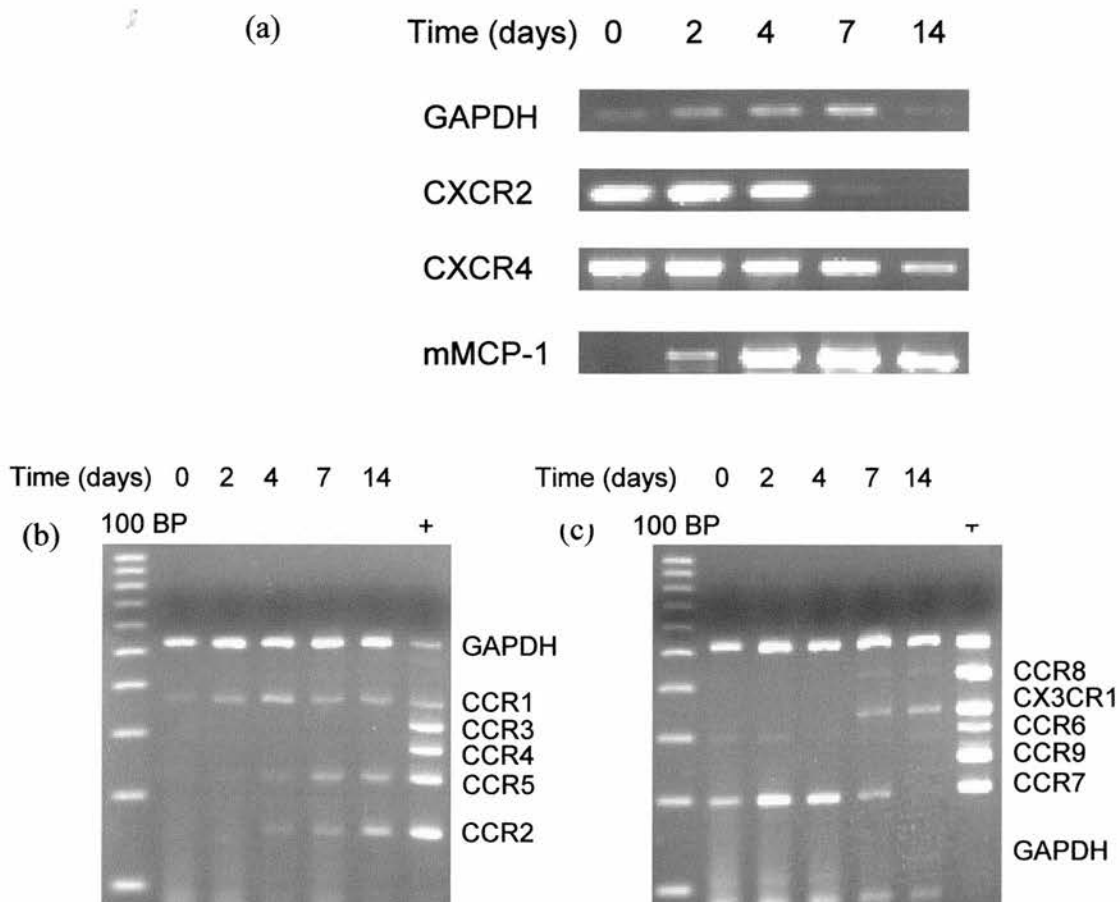
Time (days)	% MC (Leishman's)	% MC (toluidine blue)	% MMC <sub>h</sub> (mMCP-1 <sup>+</sup> )
0	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)
2	15.0 (13.0 - 17.0)	18.5 (15.0 - 27.0)	0.0 (0.0 - 0.0)
4	61.0 (58.0 - 63.0)	48.5 (45.0 - 55.0)	14.0 (11.0 - 22.0)
7	98.0 (97.0 - 99.0)	94.5 (93.0 - 96.0)	69.5 (51.0 - 78.0)
14	100.0 (100.0 - 100.0)	99.0 (98.0 - 100.0)	100.0 (100.0 - 100.0)

**Table 4.2. Cell counts from mBMMC<sup>T+</sup> cultures in experiment 4.3.**

Results show median (range) of mast cells (MC), as assessed by Leishman's, and toluidine blue staining; and of mucosal mast cell homologues (MMC<sub>h</sub>), as assessed by immunohistochemical staining for expression of mMCP-1.

500 cells were counted in cytospin preparations from each of 4 cultures at each time-point.

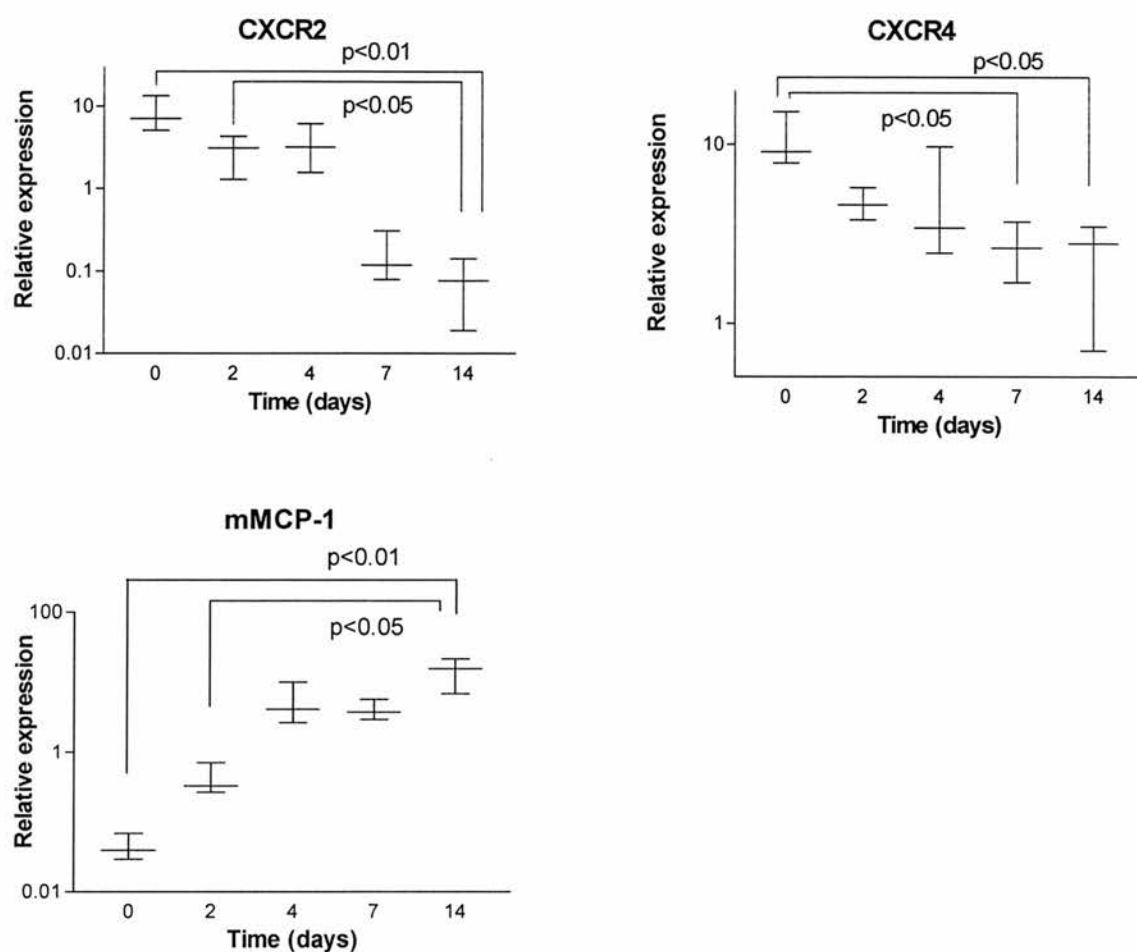
Analysis of chemokine receptor expression was generally in agreement with the results of experiment 4.2. There was down-regulation of CXCR2, CXCR4 (figure 4.3a) and CCR7 (figure 4.3c) expression with time, though CXCR4 was still detectable in mBMMC<sup>T+</sup> on day 14. Expression of CXCR2 was significantly lower on day 14 than on days 0 ( $p<0.01$ ) and 2 ( $p<0.05$ )(figure 4.4) and expression of CXCR4 on both days 7 and 14 was significantly lower than on day 0 ( $p<0.05$ )(figure 4.4).



**Figure 4.3. Expression of chemokine receptors during differentiation of mBMMC<sup>T+</sup> (MMC homologues) in experiment 4.3.**

- (a) Semi-quantitative RT-PCR showed down-regulation of expression of CXCR2 and CXCR4 as mMCP-1 expression increased.
- (b) Analysis of CCR1 - 5 using CytoXpress<sup>TM</sup> CCR set 1 mPCR kit showed expression of CCR1 at all stages of mast cell differentiation and upregulation of CCR2 and CCR5 on days 7 and 14.
- (c) Analysis of CCR6 - 9 and CX3CR1 using CytoXpress<sup>TM</sup> CCR set 2 mPCR kit showed downregulation of CCR7 and upregulation of CX3CR1 expression. Weak expression of CCR6 in several cultures, and of CCR8 mainly in day 7 and 14 cultures was also detected.

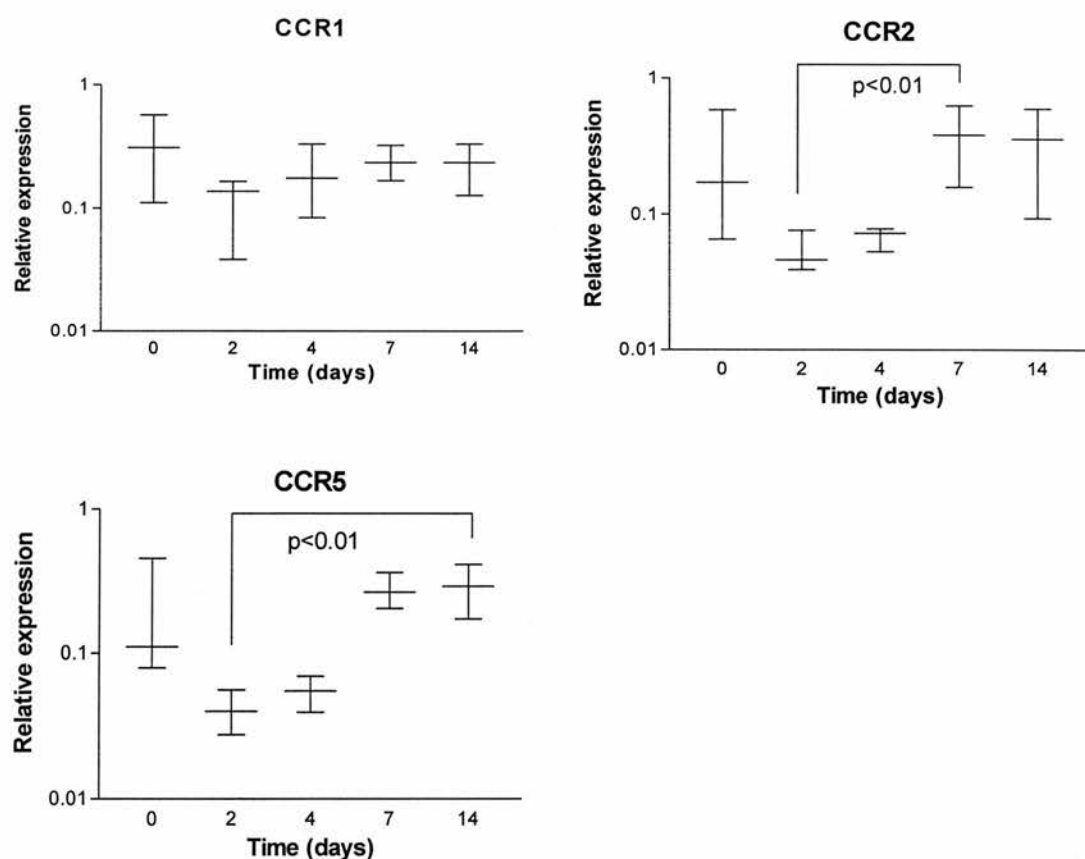
Results are representative gels from quadruplicate cultures. + - positive control.



**Figure 4.4. Relative expression of chemokine receptors CXCR2 and CXCR4, and mMCP-1 during differentiation of mBMMC<sup>T+</sup> (MMC homologues) in experiment 4.3.**

Expression of chemokine receptors and mMCP-1 was analysed over 14 days during culture of mBMMC<sup>T+</sup> from murine bone marrow cells.

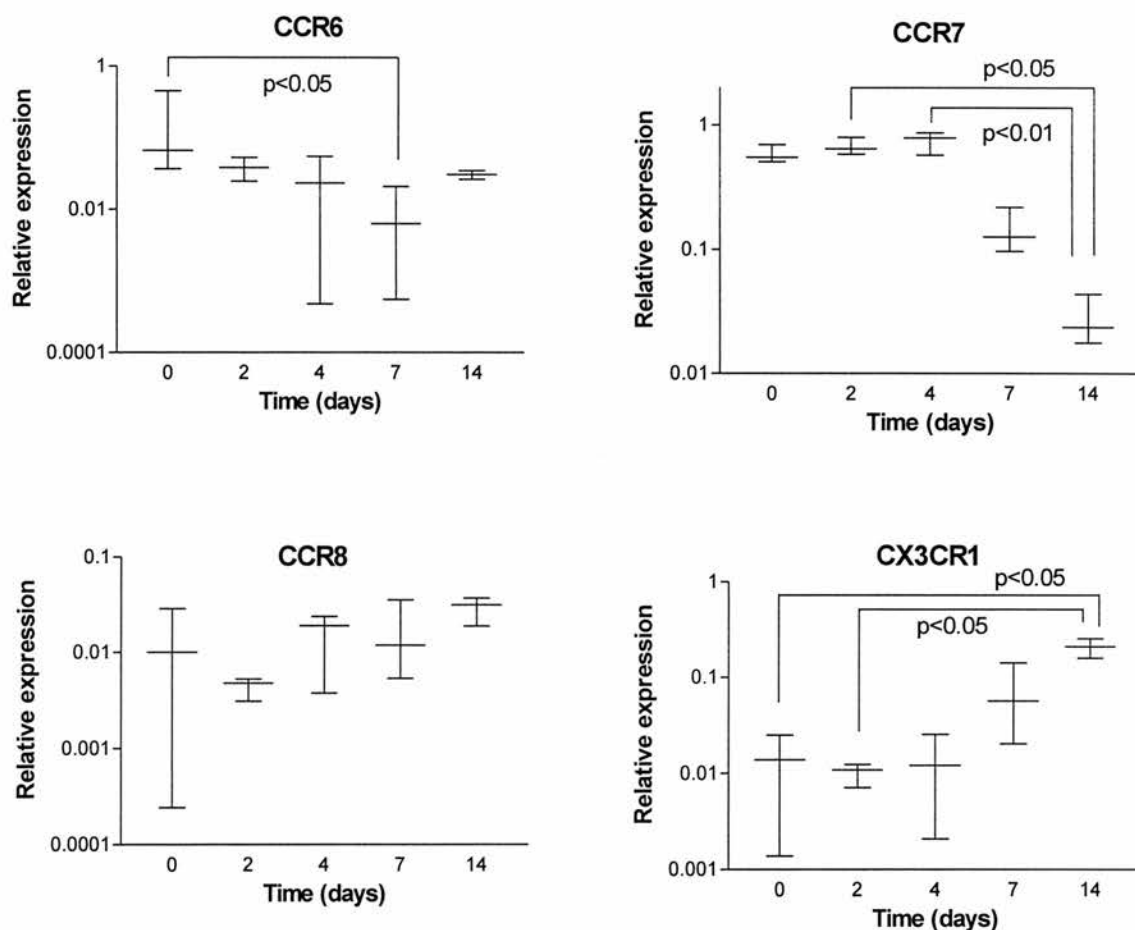
Results are shown as median (range), n=4 cultures, and statistical analysis was performed using the Kruskal-Wallis test, with Dunn's multiple comparison post-test.



**Figure 4.5. Relative expression of chemokine receptors CCR1, CCR2 and CCR5 during differentiation of mBMMC<sup>T+</sup> (MMC homologues) in experiment 4.3.**

Expression of chemokine receptors was analysed over 14 days during culture of mBMMC<sup>T+</sup> from murine bone marrow cells.

Results are shown as median (range), n=4 cultures, and statistical analysis was performed using the Kruskal-Wallis test, with Dunn's multiple comparison post-test.



**Figure 4.6. Relative expression of chemokine receptors CCR6, CCR7, CCR8 and CX3CR1 during differentiation of mBMMC<sup>T+</sup> (MMC homologues) in experiment 4.3.**

Expression of chemokine receptors was analysed over 14 days during culture of mBMMC<sup>T+</sup> from murine bone marrow cells.

Results are shown as median (range), n=4 cultures, and statistical analysis was performed using the Kruskal-Wallis test, with Dunn's multiple comparison post-test.

CCR2 and CCR5 were expressed on days 7 and 14 (figure 4.3b). However, though there was a trend towards increasing expression of CCR2 and CCR5 with time, strong expression was also detected in some day 0 samples (figure 4.5). Expression of CCR2 was significantly ( $p<0.01$ ) greater on day 7 than on day 2, and CCR5 expression was significantly ( $p<0.01$ ) greater on day 14 than on day 2 (figure 4.5). Expression of CX3CR1 also increased with time, and was significantly greater ( $p<0.05$ ) on day 14 than on days 0 and 2 (figure 4.6).

Expression of CCR1 at all time points was confirmed, (figure 4.3b), with no significant differences between cultures (figure 4.5). Expression of CCR6 was low in all samples (figure 4.3) and, as suggested by the previous experiment, there was a slight trend towards decreasing expression with time. Values were significantly lower ( $p<0.05$ ) than day 0 on day 7, but not on day 14, when weak expression was detected in all samples (figure 4.6). As described in 2.6.2, the lower intensity of this band in the positive control suggests less efficient amplification of CCR6 than of other PCR products in this kit, thus, in reality, expression relative to other receptors may be higher than shown. Low expression of CCR8 was detected mainly in day 7 and 14 cultures and there was a slight trend towards increasing expression with time (figure 4.6), but this was not significant. As shown in the previous experiment, expression of CCR3, CCR4 and CCR9 was below detectable levels in all cultures.

Since the manufacturers claimed that amplification efficiencies of each primer pair were equivalent within Biosource multiplex PCR kits, relative expression of those chemokine receptors detected could be compared. Therefore, expression of different

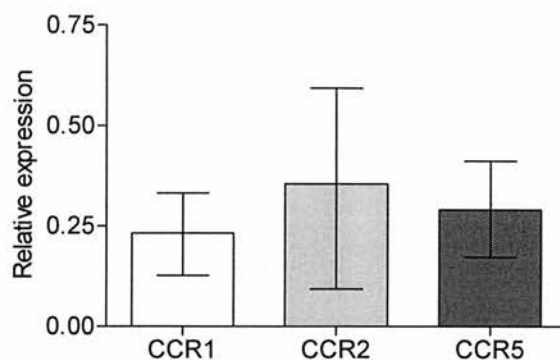


chemokine receptors in day 14 mBMMC<sup>T+</sup> was compared as, at this stage of differentiation, cultures contain a homogeneous population of cells representative of mucosal mast cells. The levels of expression of chemokine receptors analysed by different kits could not be compared, as their amplification efficiencies may be different.

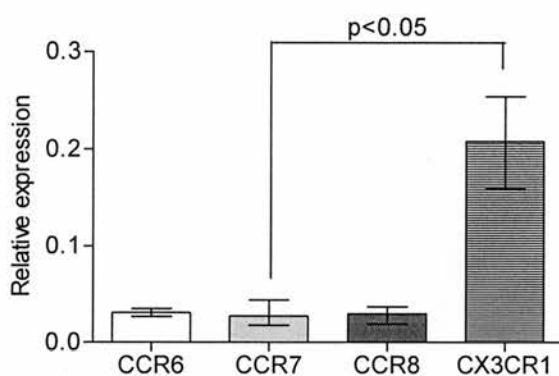
Expression of mRNA for chemokine receptors CCR1 - 5 was investigated using a CytoXpress<sup>TM</sup> CCR set 1 mPCR kit. Expression of CCR1, 2, and 5 was detected in 14 day old mBMMC<sup>T+</sup>, but no significant differences in levels of expression between any of the 3 receptors were found using the Mann-Whitney U test (figure 4.7). As stated above, expression of CCR3 and CCR4 was not detected, implying that mRNA for these chemokines was expressed at lower levels than for CCR1, 2 and 5.

mRNA for chemokine receptors CCR6 - 9 and CX3CR1 was amplified using a CytoXpress<sup>TM</sup> CCR set 2 mPCR kit. CX3CR1 was the most highly expressed receptor of this set in mBMMC<sup>T+</sup> (figure 4.7). CCR6, 7 and 8 were expressed at much lower levels, and mRNA levels of CCR7 were found to be significantly ( $p < 0.05$ ) lower than those of CX3CR1 using the Kruskal-Wallis test with Dunn's multiple comparison post-test. Expression of CCR9 was below detectable levels in mBMMC<sup>T+</sup>.

(a)



(b)



**Figure 4.7. Relative expression of chemokine receptors by MMC homologues analysed in experiment 4.3 using mPCR.**

- (a) Expression of CCR1 - 5, analysed using CytoXpress™ CCR set 1 mPCR kit. There was no significant difference between expression of CCR1, 2 and 5 using the Mann-Whitney U test, and expression of CCR3 and 4 was not detected.
- (b) Expression of CCR6 - 9 and CX3CR1, analysed using CytoXpress™ CCR set 1 mPCR kit. Expression of CX3CR1 was significantly greater ( $p < 0.05$ ) than CCR7 using the Kruskal-Wallis test with Dunn's multiple comparison post-test. Expression of CCR9 was not detected. Results are expressed as median (range),  $n=4$ .

## 4.4 EXPRESSION OF CHEMOKINE RECEPTORS IN MURINE JEJUNAL EPITHELIUM

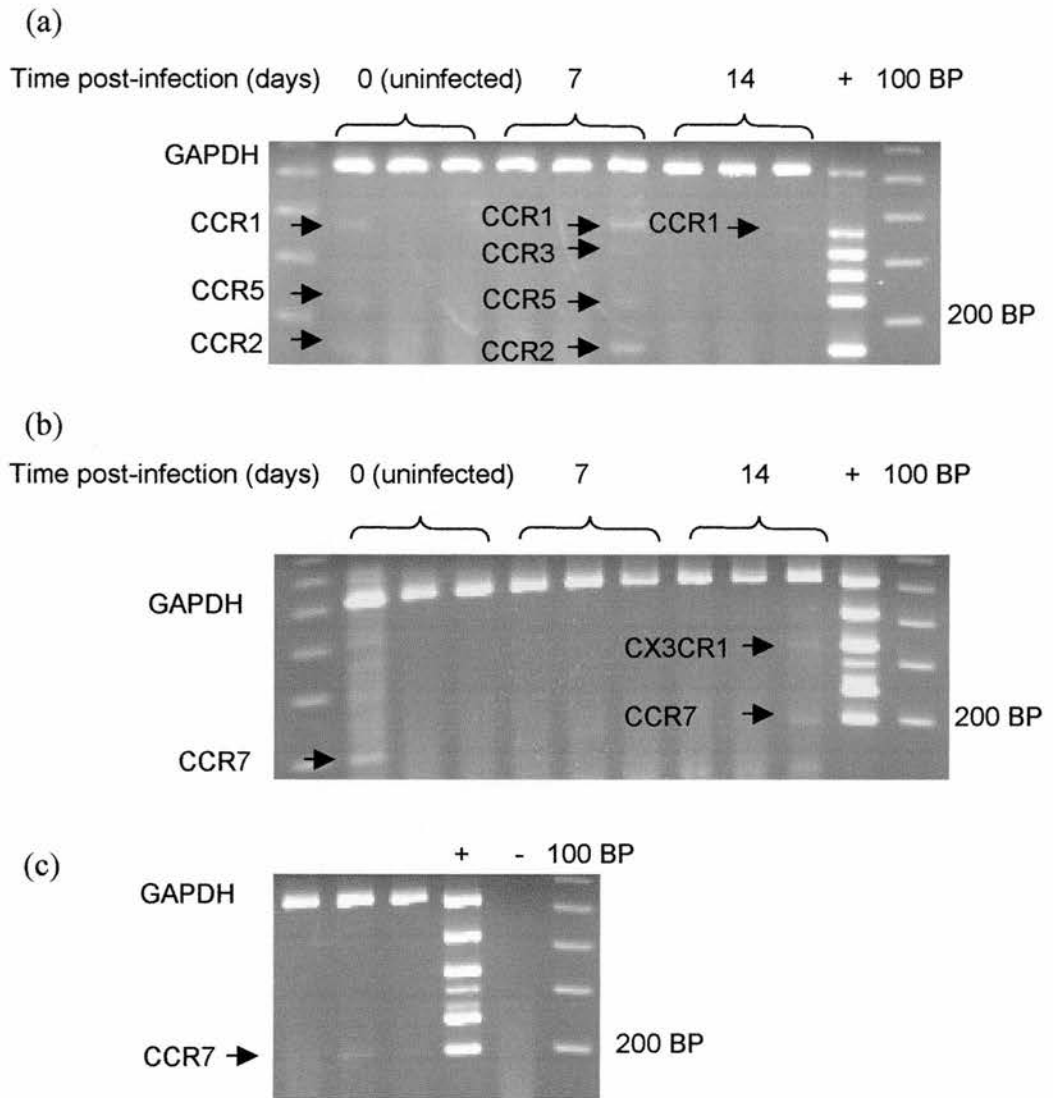
### 4.4.1 *Experimental aim and design*

Expression of transcripts for several chemokine receptors has been shown by mBMMC<sup>T+</sup>, but it is not known if these receptors are expressed by intraepithelial MMC *in vivo*. If expression of chemokine receptors by MMC was high, this might be reflected by increased expression of these receptors in intestinal epithelium on days 7 and 14 post-infection, when mast cell recruitment is at a maximum (3.2.2.2).

Expression of chemokine receptors in control (uninfected) murine jejunal epithelium, and in jejunal epithelium collected on days 7 and 14 post-*N. brasiliensis* infection for use in experiment 3.2 was therefore analysed. As in experiments 4.2 and 4.3, expression of CCR1 – 9 and CX3CR1 was analysed using mPCR (2.6), and CXCR2 and CXCR4 were analysed by RT-PCR (2.5.1 – 2.5.8). Additionally, expression was analysed in 3 samples of CMT-93 cells (2.4.4).

### 4.4.2 *Results*

Chemokine receptor expression in the majority of jejunal epithelial samples was below levels detectable by the mPCR kits under the conditions used. However, expression of mRNA for CCR1, 2 and 5 was detected in epithelium from one uninfected mouse; expression of CCR1, 2, 3 and 5 was detected in one sample collected on day 7 and expression of CCR1 only was detectable in one sample collected on day 14 post-infection with *N. brasiliensis*, using CCR set 1 (figure 4.8).



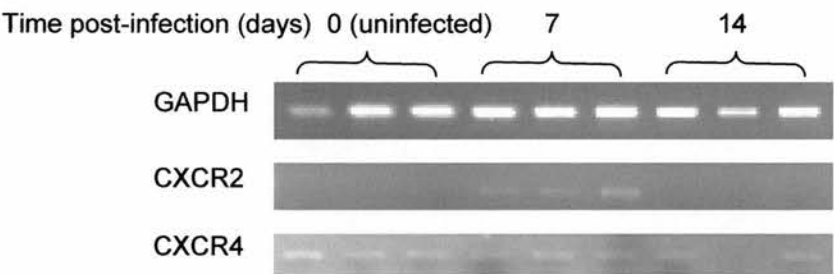
**Figure 4.8. Expression of chemokine receptors in murine jejunal epithelium during *N. brasiliensis* infection.**

- Expression of CCR1 - 5, analysed using CytoXpress™ CCR set 1 mPCR kit. CCR1, 2 and 5 were expressed in epithelium from an uninfected mouse and a mouse infected 7 days previously. Expression of CCR3 in epithelium collected on day 7, and of CCR1 in epithelium collected on day 14 post-infection was also detected.
- Expression of CCR6 - 9 and CX3CR1, analysed using CytoXpress™ CCR set 2 mPCR kit. Expression of CCR7 in uninfected epithelium, and of CCR7 and CX3CR1 in epithelium collected 14 days after infection was detected.
- Expression of CCR6 - 9 and CX3CR1 in CMT 93 epithelial cells, analysed as above. CCR7 was expressed in all three samples.

+ positive, - negative control.

Using CCR set 2, expression of CX3CR1 and CCR7 was detected in an epithelial sample collected on day 14 post-*N. brasiliensis* infection, and expression of CCR7 was also detected in epithelium from one uninfected mouse.

Expression of CCR7 was detected in all three samples of CMT-93 epithelial cells (figure 4.8) suggesting epithelial cells as a potential source of expression of this receptor, but expression of chemokine receptors analysed using CCR set 1 was below detectable levels in CMT-93 cells (not shown).



**Figure 4.9. Expression of chemokine receptors in murine jejunal epithelium during *N. brasiliensis* infection.**

Expression of CXCR4 was detected in all except one epithelial sample. CXCR2 was expressed only in epithelial samples collected on day 7 post-infection, and was below detectable levels in epithelium from uninfected mice and from mice infected 14 days previously.

Using RT-PCR, weak expression of CXCR4 was detected in all jejunal epithelial samples with the exception of one sample collected on day 14 post-infection (figure 4.9), but expression of CXCR4 was below detectable levels in CMT-93 cells (not shown). CXCR2, which was not detectable in mBMMC<sup>T+</sup>, was undetectable in CMT-93 cells (not shown) but was expressed in epithelial samples collected on day 7

post-*N. brasiliensis* infection only. This may represent an upregulation of expression on day 7 post-infection, but semi-quantitative analysis of expression would be required to confirm this. Saturation of the GAPDH signal in some samples, and the lack of detectable bands in uninfected epithelium and epithelium collected on day 14 post-infection would lead to inaccuracies in comparison of expression semi-quantitatively, therefore this was not attempted.

## 4.5 DISCUSSION

Chemokine receptors CCR1, CCR2, CCR5 and CX3CR1 were most strongly expressed by mature mBMMC<sup>T+</sup>, and experiments described in chapter 3 showed expression of several potential chemokine ligands for these receptors by intestinal epithelium, including RANTES, MIP-1 $\alpha$  and fractalkine.

Chemokine receptor expression by mBMMC<sup>T+</sup> shares similarities with expression in other MC phenotypes. IL3-dependent mBMMC, which resemble MMC in that they contain chondroitin sulphate proteoglycan (Razin *et al*, 1984; Haig *et al*, 1988), migrate towards chemokine ligands for CCR1, 2 and 5 (Taub *et al*, 1995) and express CX3CR1 (Papadopoulos *et al*, 2000), and mBMMC cultured in IL-3 and SCF express functional CCR1, 2, 3 and 5 (Oliveira & Lukacs, 2001). mBMMC cultured in IL-4 and SCF, and used to represent connective tissue MC, and freshly harvested human skin MC, also express CX3CR1 (Papadopoulos *et al*, 2000). hCBMC cultured for 4 weeks in SCF, IL-6 and IL-10, and said to represent mast cell progenitors, expressed CCR5 and CXCR4, along with CXCR2 and CCR3 which are not expressed by mBMMC<sup>T+</sup>.

Expression of the same chemokine receptors by MC of different phenotypes and cultured in the presence of different cytokines brings into question the role of MC growth factors in regulation of chemokine receptor expression. Experiment 4.2 showed TGF- $\beta_1$  to have no effect on chemokine receptor expression in mBMMC, but Oliveira & Lukacs (Oliveira & Lukacs, 2001) noted upregulation of several CC chemokine receptors by SCF. However, the same receptors are expressed by mast

cells cultured in IL-3 only (Taub *et al*, 1995), therefore chemokine receptor expression by mast cells may be regulated by several overlapping pathways or may be programmed in mature mast cells irrespective of phenotype.

mBMMC<sup>T+</sup> express receptors to chemokines frequently associated with inflammatory and allergic diseases, such as asthma and IBD (Conti *et al*, 1999; Homey & Zlotnik, 1999; Macdermott, 1999; Teran, 2000), where mast cells have a proposed role in the pathogenesis. Furthermore, the chemokine receptors expressed by mBMMC<sup>T+</sup> are consistent with those implicated in recruitment of MC in inflammatory states. Administration of met-RANTES, a specific inhibitor of CCR1 and CCR5, reduced recruitment of mast cells into the colon in a rat model of chronic colitis (Ajuebor *et al*, 2001). Also, in COPD in humans, increased MCP-1 expression by bronchiolar epithelial cells correlated with CCR2 expression on mast cells and increased numbers intraepithelially (de Boer *et al*, 2000). Low expression of MCP-1 in jejunal epithelium suggests that CCR2 is unlikely to regulate MMC recruitment following *N. brasiliensis* infection, but this receptor may control MMC recruitment in other tissues or inflammatory diseases.

CCR5 and CX3CR1, which are expressed by mBMMC<sup>T+</sup>, are also expressed by IEL (Muehlhoefer *et al*, 2000; Agace *et al*, 2000), and are thought to play a role in lymphocyte recruitment under inflammatory conditions. If comparable expression of chemokine receptors occurs by MMC *in vivo*, it would suggest recruitment mechanisms that are common in both cell types; further work should be directed



towards investigation of the expression, by mBMMC<sup>T+</sup>, of the IEL-expressed chemokine receptor, CXCR3, (Agace *et al*, 2000).

Chemokine receptors expressed by mBMMC<sup>T+</sup> are expressed by both TH1 and TH2 cells. mBMMC<sup>T+</sup> expressed mRNA for CCR5, which is expressed by TH1 cells (Rottman, 1999; Homey & Zlotnik, 1999) and weak expression of CCR8, a TH2 chemokine receptor, was also detected. Other TH2-type chemokine receptors, CCR3 and CCR4, were not expressed, so that a marked polarisation towards the pattern seen in TH1 or TH2 subsets was not present. The chemokine receptor expression profile of mBMMC<sup>T+</sup> is also similar to that reported in monocytes, which express CCR1, 2, 5 and 8; CXCR4 and CX3CR1 (Rottman, 1999).

Other notable absences from the chemokine receptor profile of mBMMC<sup>T+</sup> included CCR3 and CCR9. Expression of CCR3 has been previously shown in hCBMC cultured in IL-6, SCF and IL-10 (Ochi *et al*, 1999), human skin mast cells (Romagnani *et al*, 1999), TH2 cells, basophils and eosinophils (Ochi *et al*, 1999). Expression of the CCR3 chemokine ligand, eotaxin, has been demonstrated in the bronchial mucosa of asthmatic patients and in allergic dermatitis (Homey & Zlotnik, 1999), and, as mast cells are key players in allergic reactions, it is perhaps surprising that CCR3 expression in mBMMC<sup>T+</sup> was below detectable levels.

CCR9 transcripts were also below detectable levels in mBMMC<sup>T+</sup>. The CCR9 ligand TECK is almost exclusively expressed by small intestinal epithelium and a key role has been suggested for CCR9 in recruitment of T-cells including IEL to

mucosal sites (Wurbel *et al*, 2000; Kunkel *et al*, 2000). As previously stated, mBMMC<sup>T+</sup>, in common with IEL, express CCR5 and CX3CR1, but these receptors are thought to recruit lymphocytes in inflammatory states (Muehlhoefer *et al*, 2000; Agace *et al*, 2000). The primary involvement of CCR9 in basal lymphocyte trafficking may explain the lack of expression by mBMMC<sup>T+</sup>, as homologues of MMC which are recruited primarily in allergic reactions, and may not traffic at all. Low CCR6 expression by mBMMC<sup>T+</sup> is not surprising as this receptor is expressed mainly on memory T-cells, B cells and dendritic cells (Murdoch & Finn, 2000), and is involved in specific recruitment of Langerhan's cell precursors to the skin (Caux *et al*, 2000).

The changes in chemokine receptor expression during mast cell differentiation should be interpreted with care, as in the earlier stages of culture, a mixed population of cells is present in which there are few mast cells. Therefore whilst it is probable that receptors such as CCR7 and CXCR2, which are highly expressed in early cultures but expressed at low-to-undetectable levels in mature mBMMC<sup>T+</sup> do not play a key role in intraepithelial mast cell migration, it is not yet clear whether mast cell progenitors present in earlier cultures express these receptors. As CCR7 is expressed in mature dendritic cells (Caux *et al*, 2000) and memory and effector T-cells (Kunkel & Butcher, 2002), and is important in homing to lymph nodes, loss of this receptor from cultures as mast cell numbers increase is not surprising. However, CXCR2, which is primarily expressed by neutrophils (Rottman, 1999), is also expressed by HMC-1 cells (Lippert *et al*, 1998; Nilsson *et al*, 1999). The latter are often described as an immature mast cell phenotype, therefore CXCR2 may be

expressed by mast cell progenitors and play a role in the initial recruitment into tissues, but may be down-regulated in mature MMC. In support of this, Ochi *et al* (Ochi *et al*, 1999) found expression of CXCR2 in hPrMC but not in mature mast cells.

The same group also found expression of CXCR4 in hPrMC but not mature cells, and in our experiments, CXCR4 was down-regulated during mast cell differentiation, but still detectable in mature mBMMC<sup>T+</sup>. Therefore, CXCR4 may also be important in migration of mature cells and Juremalm *et al* (Juremalm *et al*, 2000) showed expression of CXCR4 by cultured cells resembling both immature and mature mast cell phenotypes. However, as the CXCR4 ligand SDF-1 is expressed mainly by stromal cells and not epithelium, its main role may be in recruitment of mast cell progenitors, as suggested by others (Juremalm *et al*, 2000; Lin *et al*, 2000).

Upregulation of CCR2 and 5 and CX3CR1 during mast cell differentiation indicates their probable importance in the mature phenotype, but variable expression of CCR2 and 5 in freshly isolated bone marrow cells is not in keeping with this trend. The reason for this is unknown, and may be due to the presence of a mixed cell population; further experiments to investigate chemokine receptor expression by mast cell progenitors would clarify the picture. In contrast to our findings, Ochi *et al* (Ochi *et al*, 1999) suggested down-regulation of expression of CCR5 during differentiation of hCBMC in IL-6, IL-10 and SCF. This may reflect differences in the cytokines used to culture these cell types.

Additional experimental work is required to resolve the problem of chemokine receptor expression in mast cell progenitors, and to further investigate the role of chemokine receptor expression by mast cells in intraepithelial migration; this is discussed in the final chapter.

Weak expression of mRNA for several chemokine receptors including CCR1, 2, 3, 5, 7 and CX3CR1 was detected in jejunal epithelium, and expression of CXCR4 was detected in all but one jejunal epithelial sample. However, no pattern emerged which would suggest intraepithelial MMC as the source of expression. Epithelial cells themselves may express these chemokines; in support of this, CMT-93 cells expressed CCR7 and human intestinal epithelial cells lines express CCR1 - 8 and CXCR4 (Dwinell *et al*, 2001); expression of CX3CR1 has not been investigated.

Detection of CXCR2 mRNA only in epithelium collected 7 days post-infection with *N. brasiliensis* may result from more target cDNA being present in these samples, or expression may truly be higher. If this is the case, the cellular source of expression is debatable as CXCR2 is primarily expressed by neutrophils (Rottman, 1999), which are not a major feature of the immune response to *N. brasiliensis*. However, low numbers of contaminating neutrophils were present in some epithelial samples (table 3.1). Expression of CXCR2 was not detected in mBMMC<sup>T+</sup>, making MMC a less likely source of expression. Furthermore, expression was not detected in CMT-93 epithelial cells, but low expression in other human intestinal epithelial cell lines (Dwinell *et al*, 2001), suggests epithelial cells as a possible source of this receptor. Further optimisation of the semi-quantitative RT-PCR method, or other more

quantitative methods would be required to confirm upregulation of CXCR2 in jejunal epithelium from *N. brasiliensis* infected mice.

The multiplex PCR method was useful as a screening technique to analyse expression of several chemokine receptor genes at once, and the ability to analyse relative expression of mRNAs was a useful indicator of their likely importance. However, the limitations of these kits for semi-quantitative analysis should be appreciated. Also, receptors for which mRNA was not detected may have been expressed at a level undetectable by the method used.

In conclusion, this study suggests that the chemokine receptor expression profile of mBMMC<sup>T+</sup> shares some similarities but is distinct from those previously reported in other mast cell phenotypes and IEL.

## CHAPTER 5

### ADHESION OF mBMMC TO EXTRACELLULAR MATRIX PROTEINS

#### 5.1 INTRODUCTION

In this thesis it has been speculated that chemotactic molecules may direct MMC or their precursors across the intestinal lamina propria and into the epithelium.

However, migration and location of different types of cells, including immune cells, within tissues is also regulated by other factors including the ability to adhere to the extracellular matrix (ECM)(Springer, 1994).

As described in 1.9.3 and 1.9.4, the ECM is a complex network of high molecular weight proteins. Proteins such as type I collagen, fibronectin and vitronectin make up the ECM of the intestinal lamina propria, whereas laminin and type IV collagen are components of basement membranes, which support epithelia throughout the body (Hynes, 1993; Mosher, 1993; Olson, 1993a; Olson, 1993b). Adhesion of several mast cell phenotypes to ECM proteins has been described. Murine IL-3-dependent BMMC and several murine mast cell lines (Thompson *et al*, 1989a; Dastyk *et al*, 1991; Bianchine *et al*, 1992), human skin mast cells (Columbo *et al*, 1995) (Columbo & Bochner, 2001) and HMC-1 cells (Kruger-Krasagakes *et al*, 1996) adhere to laminin, fibronectin and vitronectin; HMC-1 cells also adhere to type I and type IV collagens (Kruger-Krasagakes *et al*, 1996).

Stimulation by PMA or Fc $\epsilon$ RI cross-linking is generally required for adhesion of murine mast cells to ECM proteins (Thompson *et al*, 1989a; Dastyk *et al*, 1991), but

adhesion can also be regulated by cytokines. SCF stimulates adhesion of IL-3-dependent mBMMC to fibronectin (Dastyk & Metcalfe, 1994) and of human intestinal mast cells to fibronectin, laminin and several collagens (Lorentz *et al*, 2002). Culture of IL-3-dependent mBMMC with TGF- $\beta_1$  for up to 48 h also increased adhesion to laminin-1 (Thompson *et al*, 1990), and since this phenomenon occurred over a prolonged period of time, the mechanism was suggested to be regulation of expression of integrins, the major molecules mediating cell adhesion to the ECM.

As previously described, TGF- $\beta_1$  regulates *in vitro* differentiation of bone marrow cells into cells resembling MMC in the mouse (mBMMC<sup>T+</sup>, 2.4.1) (Miller *et al*, 1999) but their adhesion to ECM has not been studied. Previous work has shown regulation of expression of integrins during mast cell differentiation (Gurish *et al*, 1992a; Smith *et al*, 1994), therefore it was hypothesised that TGF- $\beta_1$  might also regulate adhesion of mBMMC<sup>T+</sup>, possibly by alteration of integrins expressed. Since MMC are located intraepithelially (Scudamore *et al*, 1997) our current hypothesis is that TGF- $\beta_1$  might upregulate adhesion to basement membrane proteins such as laminin, and that this might facilitate intraepithelial location.

The aim of these studies, therefore, was to investigate the adhesion of mBMMC<sup>T+</sup> to ECM proteins, and to evaluate the effect of TGF- $\beta_1$  and of mast cell activating agents on the adhesion process.

## 5.2 PRELIMINARY INVESTIGATION OF THE ADHESION OF mBMMC<sup>T+</sup> TO ECM PROTEINS

### 5.2.1 *Experimental aim and design*

Adhesion of unstimulated mBMMC<sup>T+</sup> to laminin-1, fibronectin, vitronectin and collagens type I and IV was investigated first, as previous studies have shown adhesion of mast cells to these proteins. Other work (Thompson *et al*, 1989a; Dastyk *et al*, 1991; Bianchine *et al*, 1992) suggested 20 µg/ml to be the optimum concentration for the ECM proteins examined, therefore ELISA plate wells were coated, in triplicate, with proteins at 20 µg/ml, or with 3% BSA in PBS as a negative control. Maximal adhesion of murine and human mast cells was reported after incubation for 60 min in most previous studies (Thompson *et al*, 1989a; Dastyk *et al*, 1991; Kruger-Krasagakes *et al*, 1996), therefore adhesion of mBMMC to laminin-1, fibronectin, vitronectin, type I collagen and type IV collagen was investigated after 1 or 2h to confirm the optimum timing for mBMMC<sup>T+</sup>. Adhesion assays were performed as described in 2.11.1 and 2.11.2.

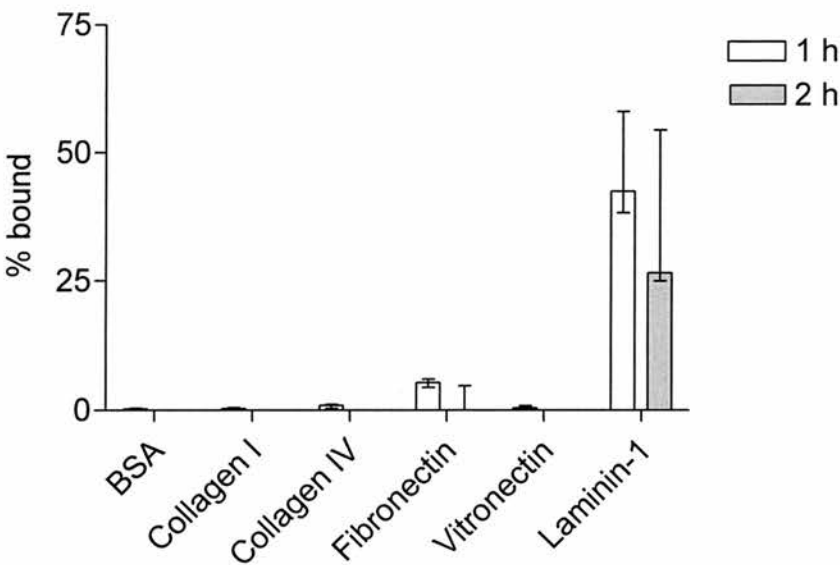
### 5.2.2 *Results*

After incubation for 1 h, mBMMC<sup>T+</sup> adhered spontaneously to laminin-1 (42.6% (38.3 - 58.1%)(median (range), n=3) and a much smaller proportion (4.3% (5.3 - 6.0%)) adhered to fibronectin (figure 5.1). After 2 h, no cells were adherent to fibronectin, and a lower number of cells (26.6% (25.0 - 54.5%)) adhered to laminin-1. mBMMC<sup>T+</sup> adherent to laminin-1 flattened and took on a polarised morphology, and a smaller proportion of mBMMC<sup>T+</sup> were flattened onto fibronectin coated wells, but did not become polarised. mBMMC<sup>T+</sup> did not adhere to vitronectin, type I



collagen or type IV collagen. The above experiment was repeated twice (once for 2 h incubation), with similar results (appendix 2, table A2.1).

In summary, mBMMC<sup>T+</sup> flattened and adhered to laminin-1 (20 µg/ml), and 60 min was confirmed as a suitable incubation time for investigation of the adhesion of mBMMC<sup>T+</sup> to ECM proteins.



**Figure 5.1. Adhesion of unstimulated mBMMC<sup>T+</sup> to extracellular matrix proteins.**

5 x 10<sup>4</sup> mBMMC<sup>T+</sup> suspended in 100 µl of culture medium (without cytokines) were incubated for 1 or 2 h in wells coated with type I collagen, type IV collagen, fibronectin, vitronectin or laminin-1 at 20 µg/ml. Wells coated with 3% BSA were also included as negative controls.

Results are expressed as median (range)(n=3 wells) of one representative experiment, which was repeated twice (once for 2 h investigations), with similar results.

### 5.3 THE EFFECT OF PMA AND SUPPLEMENTATION WITH TGF- $\beta_1$ ON ADHESION OF mBMMC TO LAMININ-1

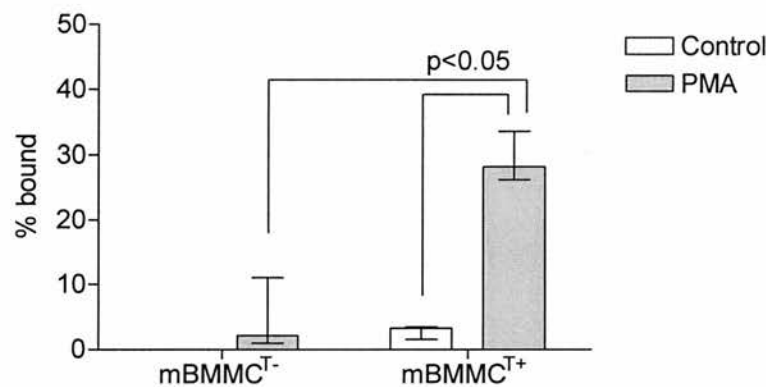
#### 5.3.1 *Experimental aim and design*

Though initial experiments showed spontaneous adhesion of mBMMC<sup>T+</sup> to laminin-1, this was inconsistent, with subsequent experiments showing more variable results (data not shown). Previous studies (Thompson *et al*, 1990) showed a requirement for cell activation using agents such as PMA for adhesion of mast cells to laminin, therefore the effect of PMA on adhesion of mBMMC<sup>T+</sup> to laminin-1 was investigated.

As discussed in 5.1, we also wished to investigate the effect of supplementing with TGF- $\beta_1$  on adhesion of mBMMC to laminin, therefore adhesion of mBMMC<sup>T-</sup> (cultured without TGF- $\beta_1$ ) and mBMMC<sup>T+</sup> (cultured with TGF- $\beta_1$ ) to laminin-1 (20  $\mu$ g/ml) was examined in the presence or absence of 50 ng/ml PMA, which was added immediately after loading cells into wells. This concentration of PMA was shown by others to stimulate adhesion of IL-3-dependent mBMMC to ECM proteins (Thompson *et al*, 1989a; Dastyh *et al*, 1991). The possible requirement for anti-TGF- $\beta_1$  in culture of mBMMC<sup>T-</sup> was investigated in 2.4.2, where it was concluded that low numbers of mMCP-1<sup>+</sup> cells were present in mBMMC<sup>T-</sup> cultured without antibody. Therefore, mBMMC<sup>T-</sup> for this and subsequent experiments were cultured without anti-TGF- $\beta_1$  antibody, and numbers of mMCP-1<sup>+</sup> cells were generally below 2%.

### 5.3.2 Results

Addition of PMA significantly ( $p<0.05$ ) increased adhesion of  $\text{mBMMC}^{\text{T}+}$  to laminin-1 from 3.3% (1.6 - 3.5%) (median (range),  $n=4$ ) to 28.2% (26.6 - 33.6%) (figure 5.2). In contrast, adhesion of both control (0.0% (0.0 - 0.0%)) and PMA-stimulated (2.2% (1.0 - 11.0%))  $\text{mBMMC}^{\text{T}-}$  to laminin-1 was low. Adhesion of PMA-stimulated  $\text{mBMMC}^{\text{T}+}$  to laminin-1 was significantly higher ( $p<0.05$ ) than that of  $\text{mBMMC}^{\text{T}-}$ . Adhesion of both  $\text{mBMMC}^{\text{T}-}$  and  $\text{mBMMC}^{\text{T}+}$  to BSA was  $<1\%$  (not shown).



**Figure 5.2. Adhesion of control and PMA (50 ng/ml)-stimulated  $\text{mBMMC}^{\text{T}-}$  and  $\text{mBMMC}^{\text{T}+}$  to laminin-1 (20  $\mu\text{g}/\text{ml}$ ).**

PMA significantly ( $p<0.05$ ) increased adhesion of  $\text{mBMMC}^{\text{T}+}$  to laminin-1, and the proportion of  $\text{mBMMC}^{\text{T}+}$  adherent to laminin-1 was significantly higher ( $p<0.05$ ) than that of  $\text{mBMMC}^{\text{T}-}$ .

Results are expressed as median (range) of one experiment,  $n=4$  wells.

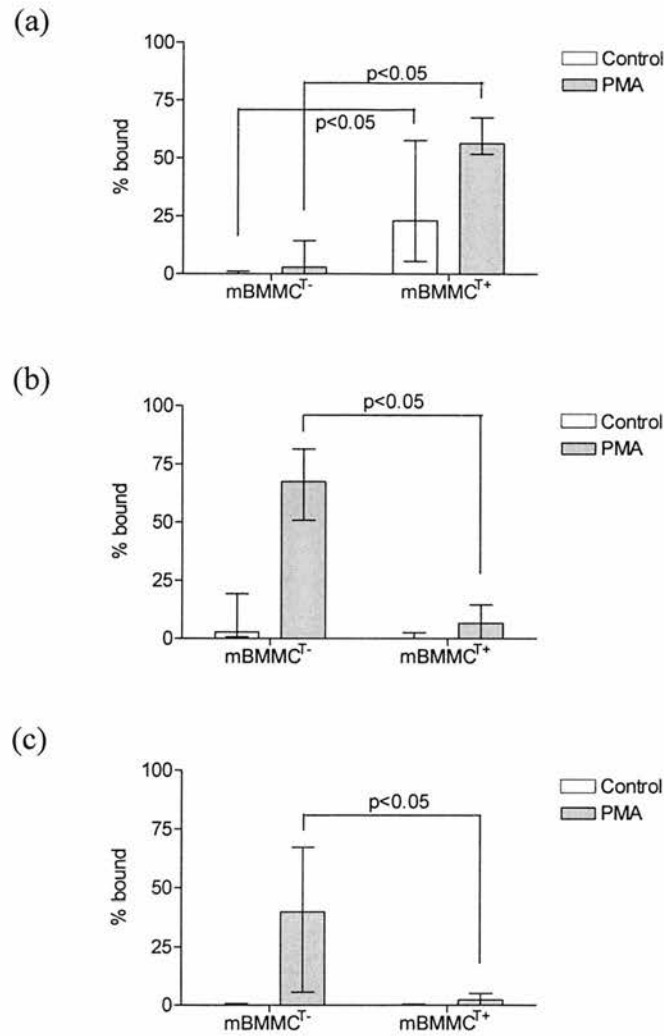
## 5.4 THE EFFECT OF TGF- $\beta_1$ ON ADHESION OF mBMMC TO ECM PROTEINS

### 5.4.1 *Experimental aim and design*

As mBMMC<sup>T-</sup> showed little adhesion to laminin-1, the effect of mBMMC culture with and without TGF- $\beta_1$  on adhesion to laminin-1 and other ECM proteins fibronectin, vitronectin, type I collagen and type IV collagen was further investigated. Proteins were coated at 20  $\mu\text{g/ml}$  (2.11.1), and adhesion of unstimulated and PMA-stimulated cells was investigated after incubation for 1 h, as described in 2.11.2. After this experiment, optimisation experiments were set up to confirm that the concentrations of protein solutions used to coat wells (5.5), and the PMA concentration used resulted in maximum cell adhesion (5.6); and to confirm the validity of the  $\beta$ -hexoseaminidase assay for estimation of adhesion of mast cells to ECM proteins (5.7). The experiment was then repeated 3 times using cells from different cultures.

### 5.4.2 *Results*

Results, expressed as median (range) of the medians from each of 4 experiments, showed that a proportion (23.0% (5.5 - 57.7%)) of mBMMC<sup>T+</sup> adhered spontaneously to laminin-1, but there was much variability between cultures (figure 5.3a; appendix 2, table A2.2). Adhesion of mBMMC<sup>T+</sup> to laminin-1 increased to 56.4% (51.8 - 67.4%) following PMA stimulation (figure 5.3a), and adhesion of both control (unstimulated) and PMA-stimulated populations of mBMMC<sup>T+</sup> was

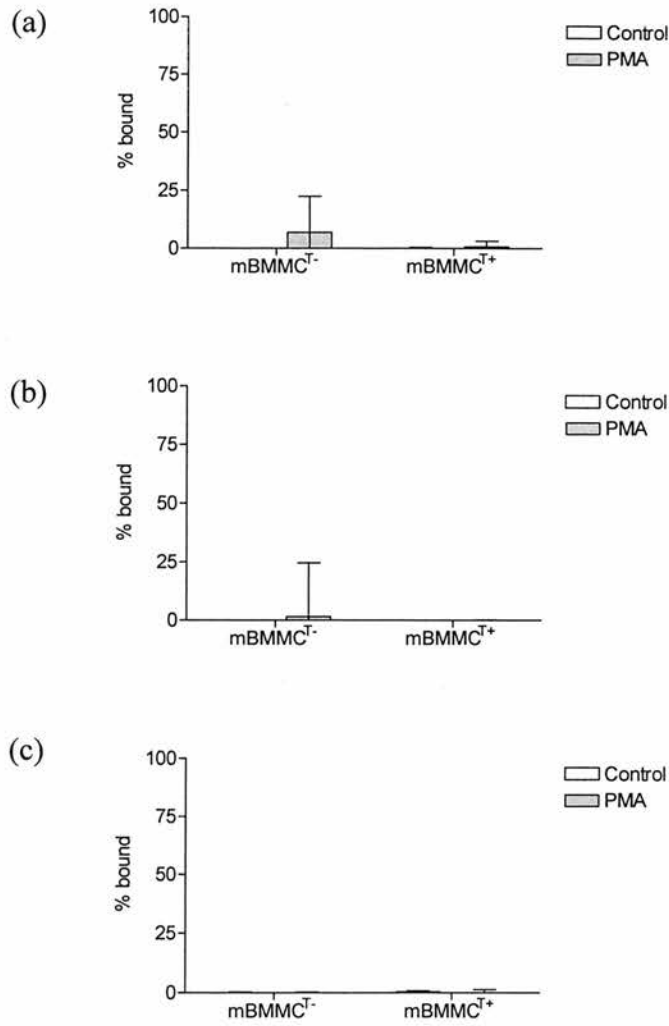


**Figure 5.3. Adhesion of unstimulated (control) and PMA-stimulated mBMMC<sup>T+</sup> and mBMMC<sup>T-</sup> to (a) laminin-1, (b) fibronectin and (c) vitronectin.**

mBMMC<sup>T+</sup> grown in the presence of TGF- $\beta_1$  adhered to laminin-1, but not to fibronectin or vitronectin, whereas mBMMC<sup>T-</sup> grown in the absence of TGF- $\beta_1$  adhered poorly to laminin-1, but adhered to fibronectin and vitronectin following PMA-stimulation.

Adhesion of both control and PMA-stimulated mBMMC<sup>T+</sup> to laminin was significantly greater ( $p < 0.05$ ) than adhesion of equivalently-stimulated mBMMC<sup>T-</sup>. Also, adhesion of mBMMC<sup>T-</sup> to fibronectin and vitronectin was significantly greater ( $p < 0.05$ ) than adhesion of PMA-stimulated mBMMC<sup>T+</sup>.

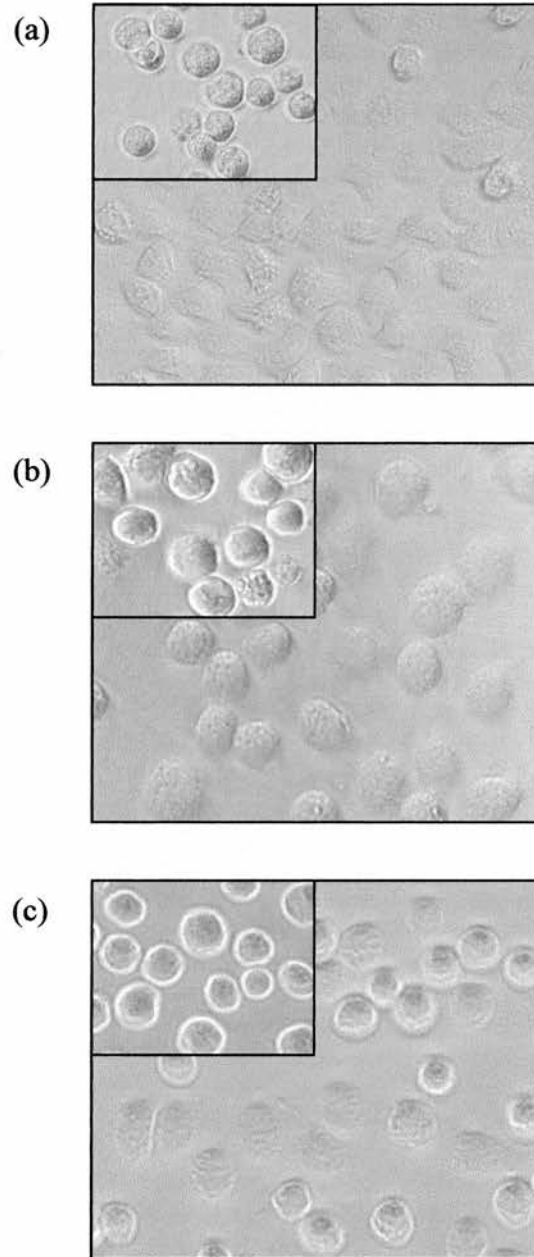
Results are expressed as median (range) of the medians from 4 separate experiments, each performed in quadruplicate wells and using cells from 4 different cultures (appendix 2, table A2.2).



**Figure 5.4. Adhesion of unstimulated (control) and PMA-stimulated mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> to (a) type I collagen, (b) type IV collagen and (c) BSA.**

mBMMC<sup>T-</sup> adhered to type I collagen in two experiments, and type IV collagen in one experiment (see appendix 2, table A2.3), but this was inconsistent, therefore not statistically significant. Adhesion of both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> to BSA was <2% in all experiments.

Results are median (range) of the medians from 4 separate experiments, each performed in quadruplicate wells and using cells from 4 different cultures.



**Figure 5.5. Adhesion of PMA-stimulated mBMMC to ECM proteins.**

mBMMC<sup>T+</sup> adhered to laminin-1 (a), the inset shows the lack of adhesion of mBMMC<sup>T-</sup> on the same surface.

mBMMC<sup>T-</sup> adhere to fibronectin (b) and vitronectin (c). Insets show the lack of adhesion of mBMMC<sup>T+</sup> on these surfaces.

significantly greater ( $p < 0.05$ ) than that of equivalently-stimulated  $\text{mBMMC}^{\text{T}^-}$ . A small proportion (6.7% (0.0 - 14.5%)) of  $\text{mBMMC}^{\text{T}^+}$  also adhered to fibronectin following PMA-stimulation.

As previously shown (experiment 5.3) neither control nor PMA-stimulated  $\text{mBMMC}^{\text{T}^-}$  adhered to laminin-1. However, control (unstimulated)  $\text{mBMMC}^{\text{T}^-}$  exhibited low (2.9% (0.8 - 19.2%)) spontaneous adhesion to fibronectin (figure 5.3b; appendix 2, table A2.2) and, after PMA stimulation,  $\text{mBMMC}^{\text{T}^-}$  adhered to fibronectin (67.5% (51.0 - 81.5%)) and vitronectin (40.2% (5.5 - 67.3%)) at levels which were significantly greater ( $p < 0.05$ ) than those observed for  $\text{mBMMC}^{\text{T}^+}$  (figure 5.3b and c; appendix 2, table A2.2). PMA-stimulated  $\text{mBMMC}^{\text{T}^-}$  also adhered to collagens in some experiments (figure 5.4; appendix 2, table A2.3), but this was variable, and there were no significant differences between adhesion of  $\text{mBMMC}^{\text{T}^-}$  and  $\text{mBMMC}^{\text{T}^+}$  to type I or type IV collagen.  $\text{mBMMC}^{\text{T}^+}$  adherent to laminin-1 flattened and took on a more polarised morphology (figure 5.5), and  $\text{mBMMC}^{\text{T}^-}$  flattened onto wells coated with fibronectin or vitronectin. In all experiments,  $\text{mBMMC}$  adhesion to BSA was  $< 2\%$  (appendix 2, table A2.3).

## 5.5 CONFIRMATION OF OPTIMUM COATING CONCENTRATIONS OF ECM PROTEINS

### 5.5.1 *Experimental aim and design*

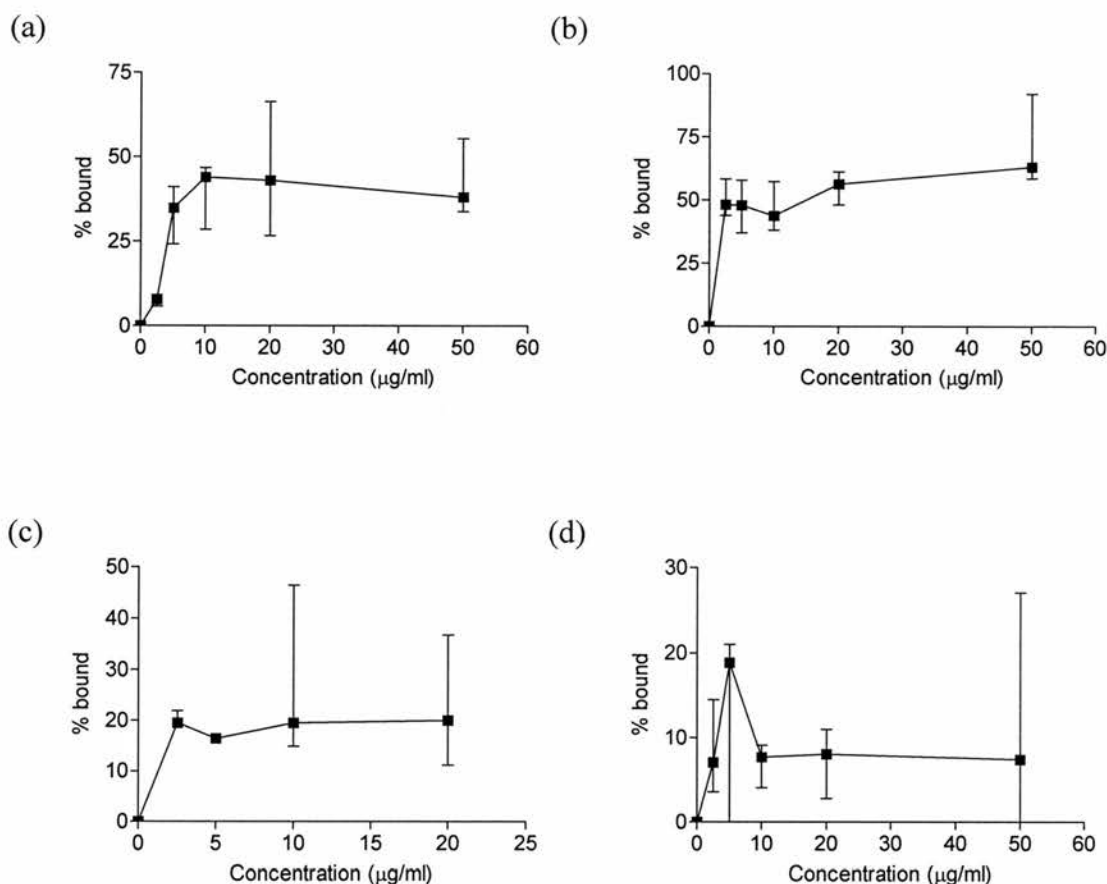
To confirm that concentrations of ECM protein solutions used for coating of wells in experiment 5.4 resulted in maximal cell adhesion, dose-response experiments were performed. PMA (50 ng/ml)-stimulated  $\text{mBMMC}^{\text{T}^+}$  were incubated (in



quadruplicates) in wells coated with 2.5, 5, 10, 20 or 50  $\mu\text{g/ml}$  of laminin-1, and PMA-stimulated mBMMC<sup>T-</sup> were incubated (in quadruplicate) in wells coated with 0.5, 1, 2, 5, 10, 20 or 50  $\mu\text{g/ml}$  of fibronectin; 2.5, 5, 10 or 20  $\mu\text{g/ml}$  of vitronectin or 2.5, 5, 10, 20 or 50  $\mu\text{g/ml}$  of type I collagen. Wells coated with BSA served as "0  $\mu\text{g/ml}$ " controls. Cell adhesion was measured as described in 2.11.2.

### 5.5.2 Results

A high proportion of both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> adhered to ECM proteins even at the lowest coating concentrations (figure 5.6) and maximal adhesion of mBMMC<sup>T+</sup> to laminin-1 was obtained on wells coated with 20  $\mu\text{g/ml}$ . Maximum adhesion of mBMMC<sup>T-</sup> to fibronectin was obtained on wells coated with 50  $\mu\text{g/ml}$ , but high (89% of maximum) adhesion was obtained on wells coated with 20  $\mu\text{g/ml}$ , therefore 20  $\mu\text{g/ml}$  of both laminin-1 and fibronectin was considered acceptable for further experiments. Adhesion of mBMMC<sup>T-</sup> to vitronectin reached a maximum at 10  $\mu\text{g/ml}$ , therefore this concentration was used for further experiments due to the high cost of this reagent. Adhesion of mBMMC<sup>T-</sup> to type I collagen appeared to peak when coating was at 5  $\mu\text{g/ml}$ , but was variable, therefore 20  $\mu\text{g/ml}$  was considered acceptable for coating in further experiments. These results show that mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> adhere to ECM proteins when coated at similar concentrations to those required for adhesion of IL-3-dependent mBMMC (Thompson *et al*, 1989a; Dastyk *et al*, 1991; Bianchine *et al*, 1992).



**Figure 5.6. The effect of varying concentrations of ECM proteins on adhesion of PMA-stimulated mBMMC.**

Adhesion of mBMMC<sup>T+</sup> to laminin-1(a), and of mBMMC<sup>T-</sup> to fibronectin (b), vitronectin (c) and type I collagen (d).

These results confirm that there is close to maximum adhesion of mBMMC on wells coated with 20 μg/ml laminin-1, fibronectin, vitronectin or type I collagen.

Results shown are median (range) of one experiment, n=4 wells.

## 5.6 OPTIMISATION OF PMA TREATMENT

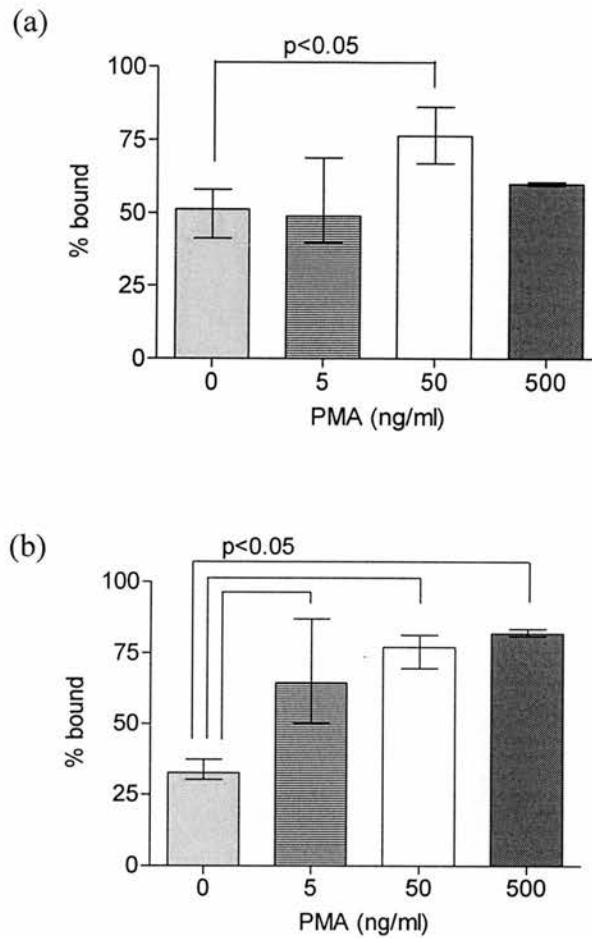
### 5.6.1 *Experimental aim and design*

Previous studies (Thompson *et al*, 1990; Dastyh *et al*, 1991) reported the use of 50 ng/ml PMA to stimulate adhesion of IL-3-dependent mBMMC to laminin and fibronectin, therefore this concentration was used to stimulate adhesion of mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> to ECM proteins. To confirm, retrospectively, that this concentration of PMA stimulated maximum adhesion, titration experiments were set up.

Adhesion of mBMMC<sup>T-</sup> to fibronectin, and of mBMMC<sup>T+</sup> to laminin-1 was investigated as described in 2.11.1 and 2.11.2. mBMMC were incubated for 60 min in the presence of 0, 5, 50 or 500 ng/ml of PMA, which was added immediately after loading of cells into coated wells.

### 5.6.2 *Results*

High spontaneous adhesion, particularly of mBMMC<sup>T+</sup> to laminin-1, in this experiment made it difficult to assess the effect of PMA on adhesion. However, use of PMA at 50 ng/ml significantly ( $p < 0.05$ ) increased adhesion of mBMMC<sup>T+</sup> to laminin-1 (figure 5.7). Spontaneous adhesion of mBMMC<sup>T-</sup> to fibronectin was lower, and addition of PMA at final concentrations of  $\geq 5$  ng/ml significantly ( $p < 0.05$ ) increased adhesion (figure 5.7). In a small pilot experiment, vehicle controls for PMA (appendix 1) were shown to have no effect on adhesion of mBMMC<sup>T+</sup> to laminin-1 or of mBMMC<sup>T-</sup> to fibronectin (data not shown).



**Figure 5.7. The effect of varying concentration of PMA on adhesion of mBMMC to ECM proteins (20  $\mu$ g/ml).**

Graphs show (a) adhesion of mBMMC<sup>T+</sup> to laminin-1 and (b) adhesion of mBMMC<sup>T+</sup> to fibronectin.

(a) Addition of 50 ng/ml PMA significantly ( $p < 0.05$ ) increased numbers of mBMMC<sup>T+</sup> adherent to laminin-1.

(b) Adhesion of mBMMC<sup>T+</sup> to fibronectin was significantly ( $p < 0.05$ ) increased by addition of  $\geq 5$  ng/ml PMA.

Results are expressed as median (range) of one experiment ( $n=4$  wells).

These results suggest that adhesion of mBMMC<sup>T-</sup> to fibronectin, and of mBMMC<sup>T+</sup> to laminin-1 is maximally stimulated by concentrations of PMA that are similar to those required to stimulate adhesion of IL-3-dependent mBMMC to these ECM proteins (Thompson *et al*, 1989a; Dastyk *et al*, 1991).

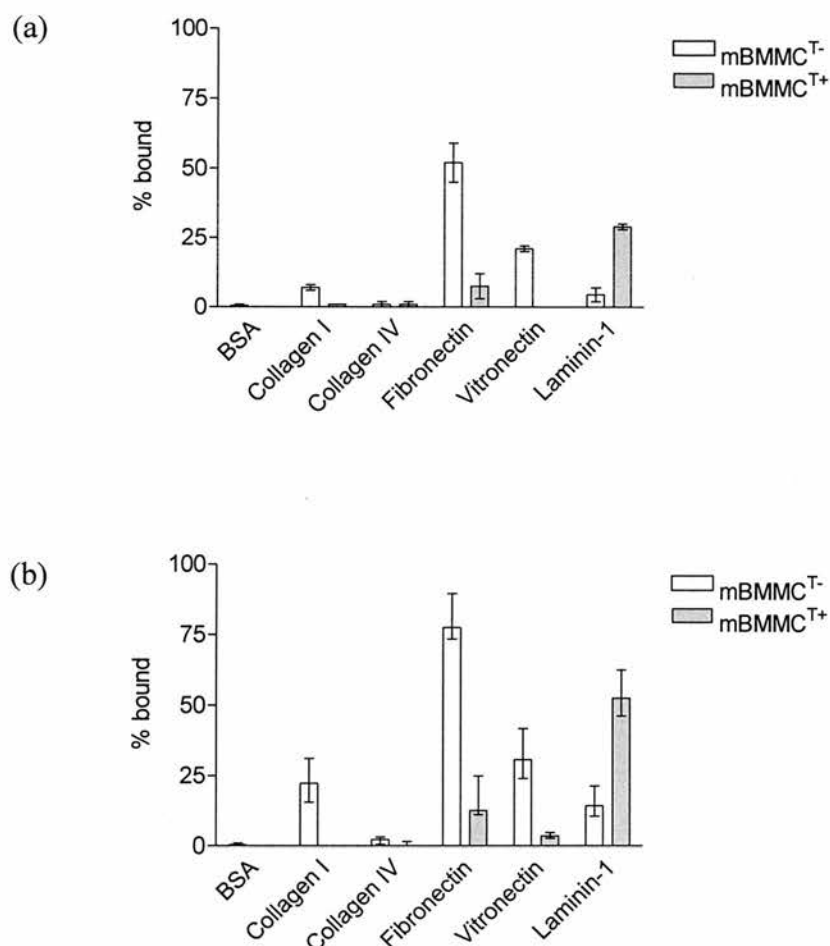
## 5.7 COMPARISON OF MANUAL COUNTING METHODS WITH THE $\beta$ -HEXOSEAMINIDASE ASSAY METHOD OF ESTIMATION OF mBMMC ADHESION TO ECM PROTEINS

### 5.7.1 *Experimental aim and design*

In order to validate the  $\beta$ -hexoseaminidase assay as a method for measurement of cell adhesion to ECM, an experiment was performed, in parallel with the conventional enzyme assay, in which adherent cells were counted manually. Adhesion of PMA-stimulated mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> to ECM proteins (as in experiment 5.4) was measured in duplicate wells by manual counting after incubation for 60 min. After removal of non-adherent cells, 200  $\mu$ l of trypsin-EDTA was added to facilitate removal of adherent cells, and incubated for 20 min at 37°C. Cells were then resuspended by gentle agitation, and 10  $\mu$ l of the cell suspension counted using an improved Neubauer haemocytometer.

### 5.7.2 *Results*

A lower number of cells adherent to laminin-1, type I collagen, fibronectin and vitronectin were counted manually compared to estimates using the  $\beta$ -hexoseaminidase assay. However, the trends in each data set using manual counts for adhesion of mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> were comparable to those estimated using the  $\beta$ -hexoseaminidase assay (figure 5.8). It was not possible to compare



**Figure 5.8. Validation of the  $\beta$ -hexoseaminidase assay.**

Comparison of (a) manual counting of duplicate wells and (b) use of the  $\beta$ -hexoseaminidase assay (quadruplicate wells) to enumerate PMA-stimulated mBMMC adherent to ECM protein-coated wells.

Although numbers of adherent cells counted by the  $\beta$ -hexoseaminidase assay were higher than those counted manually, similar trends are seen using both methods.

Results are median (range), (a)  $n=2$  wells and (b)  $n=4$  wells.

estimations of adherent cell numbers after manual counting and use of the  $\beta$ -hexoseaminidase assay statistically as adhesion by manual counting was assessed in duplicate only. However, as the trends were similar, it was concluded that, under these conditions, the  $\beta$ -hexoseaminidase assay provided a valid estimate of mast cell adhesion.

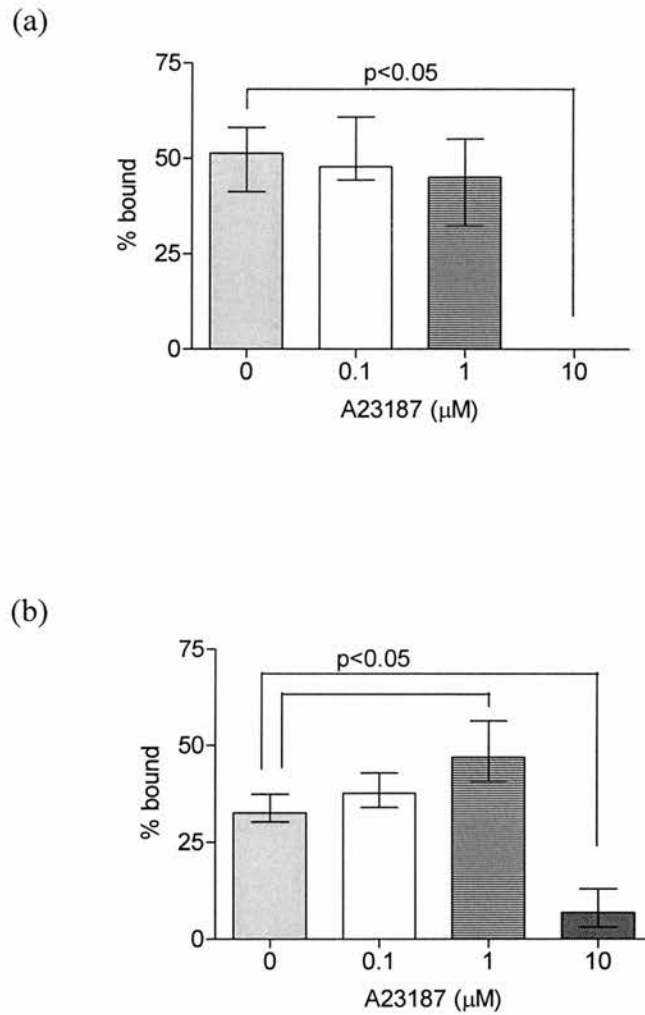
## 5.8 OPTIMISATION OF CALCIUM IONOPHORE A23187 TREATMENT

### 5.8.1 *Experimental aim and design*

Previous work (Thompson *et al*, 1990; Dastyh & Metcalfe, 1994) showed stimulation of adhesion of IL-3-dependent mBMMC to laminin-1 or fibronectin by addition of 0.1  $\mu$ M calcium ionophore A23187. However, preliminary experiments established that a 1  $\mu$ M concentration of ionophore was required for stimulation of increased adhesion of mBMMC<sup>T+</sup> to laminin-1, or of mBMMC<sup>T-</sup> to fibronectin. Therefore a titration experiment was set up to confirm that this concentration of calcium ionophore stimulated maximum adhesion, in preparation for further experiments investigating mechanisms stimulating adhesion of mBMMC to ECM proteins (5.9). Adhesion of mBMMC<sup>T-</sup> to fibronectin, and of mBMMC<sup>T+</sup> to laminin-1 was investigated as described in 2.11.1 and 2.11.2. mBMMC were incubated for 1 h in the presence of 0, 0.1, 1 or 10  $\mu$ M calcium ionophore A23187, which was added immediately after loading of cells into coated wells.

### 5.8.2 *Results*

Calcium ionophore A23187, when used at 0.1 or 1  $\mu$ M did not upregulate adhesion of mBMMC<sup>T+</sup> to laminin-1 above control values in this experiment (figure 5.9).



**Figure 5.9. The effect of varying concentration of calcium ionophore A23187 on adhesion of mBMMC to ECM proteins.**

Graphs show (a) adhesion of mBMMC<sup>T+</sup> to laminin-1 and (b) adhesion of mBMMC<sup>T-</sup> to fibronectin.

(a) Adhesion of mBMMC<sup>T+</sup> to laminin-1 was significantly reduced ( $p<0.05$ ) by addition of 10 μM A23187.

(b) Addition of 1 μM A23187 significantly increased ( $p<0.05$ ) numbers of mBMMC<sup>T-</sup> adherent to fibronectin, but addition of 10 μM A23187 resulted in a significant decrease ( $p<0.05$ ) in adherent mBMMC<sup>T-</sup>.

Results are expressed as median (range), n=4 wells.



However, this was probably due to the high spontaneous adhesion, since in other experiments where there was lower spontaneous adhesion (5.9; appendix 2, table A2.4), adhesion of mBMMC<sup>T+</sup> to laminin-1 was increased by treatment with 1  $\mu$ M A23187. Cells adherent to laminin-1 in the presence of A23187 did not undergo the shape change seen in spontaneously adherent cells, indicating a different mechanism of adhesion. Addition of 1  $\mu$ M A23187 to mBMMC<sup>T-</sup> significantly ( $p < 0.05$ ) increased adhesion to fibronectin (figure 5.9), but adherent cells did not flatten onto wells. Addition of 10  $\mu$ M A23187 to both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> significantly ( $p < 0.05$ ) reduced numbers of cells adherent to ECM proteins, this was presumed to be due to a cytotoxic effect. In a small pilot experiment, vehicle controls for A23187 (appendix 1) were shown to have no effect on adhesion of mBMMC<sup>T+</sup> to laminin-1 or of mBMMC<sup>T-</sup> to fibronectin (data not shown).

Results of this experiment and other experiments (5.9) suggest that 1  $\mu$ M calcium ionophore A23187 stimulates maximum adhesion of mBMMC to ECM proteins.

## 5.9 REGULATION OF THE ADHESION of mBMMC TO ECM PROTEINS

### 5.9.1 *Experimental aim and design*

Other published studies (Thompson *et al*, 1990; Dastyk *et al*, 1991) showed that adhesion of mast cell phenotypes to ECM proteins was regulated by Fc $\epsilon$ RI cross-linking or by addition of calcium ionophore A23187. Therefore, the primary aim of these experiments was to determine if mBMMC<sup>T+</sup>, as MMC homologues, could be stimulated to adhere to laminin-1 by similar mechanisms. Regulation of adhesion of

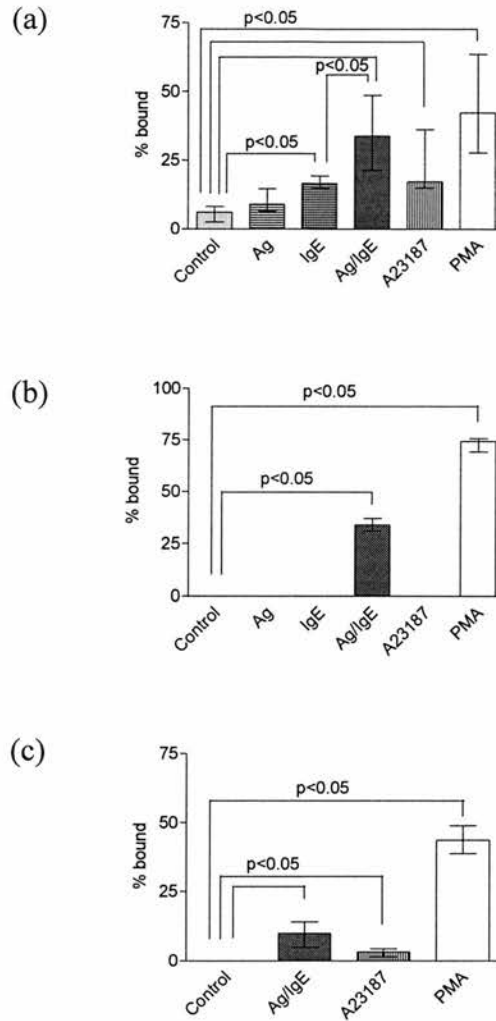
mBMMC<sup>T-</sup> was also investigated, although an *in vivo* homologue of this mast cell phenotype has not been identified.

The effects of IgE cross-linking and of calcium ionophore A23187 on adhesion of mBMMC<sup>T+</sup> laminin, and of mBMMC<sup>T-</sup> to fibronectin and vitronectin was analysed in quadruplicate using method 2.11.4.

### 5.9.2 Results

There was a trend in that numbers of mBMMC<sup>T+</sup> adherent to laminin-1 were increased above that seen in controls (unstimulated mBMMC<sup>T+</sup>) by treatment with Ag/IgE in four out of five experiments (appendix 2, table A2.4), and with calcium ionophore A23187 in four experiments using cells from separate cultures. In one experiment (appendix 2, table A2.4, experiment 2; figure 5.10) this increase was statistically significant following stimulation by both Ag/IgE and calcium ionophore. Cells adherent to laminin-1 after Ag/IgE treatment flattened and took on a polarised appearance, but A23187 treated cells retained a rounded shape. Increased adhesion after Ag/IgE or A23187 treatment was less easily demonstrable where there was high spontaneous adhesion of mBMMC<sup>T+</sup> (appendix 2, table A2.4, experiments 3, 4 and 5). Also, there was some variability in adhesion of Ag/IgE treated cells, where decreased adhesion was detected in one experiment (appendix 2, table A2.4, experiment 3). The possible reasons for this are addressed in 5.10 below.

Adhesion of mBMMC<sup>T-</sup> to fibronectin was increased by Ag/IgE in three separate experiments using cells from different cultures (figure 5.10, table A2.5), this increase was statistically significant in one experiment (figure 5.10; table A2.5, experiment



**Figure 5.10. Signals stimulating adhesion of mBMMC to ECM proteins.**

- (a) Adhesion of mBMMC<sup>T+</sup> to laminin-1 was significantly ( $p < 0.05$ ) increased by treatment with Ag/IgE and calcium ionophore A23187.
- (b) Adhesion of mBMMC<sup>T-</sup> to fibronectin was significantly ( $p < 0.05$ ) increased by Ag/IgE treatment, but no response to A23187 was seen in this experiment. However, this treatment did increase adhesion of mBMMC<sup>T-</sup> to fibronectin in other experiments (appendix 2, table A2.5).
- (c) Ag/IgE significantly ( $p < 0.05$ ) increased adhesion of mBMMC<sup>T-</sup> to vitronectin; a more substantial increase in adhesion was shown in a pilot experiment performed in triplicate (appendix 2, table A2.6). A small but significant ( $p < 0.05$ ) increase in adhesion of mBMMC<sup>T-</sup> to vitronectin was also seen after treatment with A23187.

Results are median (range) of one experiment ( $n=4$  wells). The results of repeat experiments are shown in appendix 2, tables A2.4 - A2.6.

3). Adhesion of mBMMC<sup>T-</sup> to fibronectin was also increased by A23187 in one experiment (table A2.5, experiment 2), but this increase was not statistically significant, and was not found in the repeat experiment (figure 5.10; table A2.5, experiment 3). However, 1  $\mu$ M A23187 significantly ( $p < 0.05$ ) increased adhesion of mBMMC to fibronectin in the dose-response experiment (5.8). An increase in adhesion of mBMMC<sup>T-</sup> to vitronectin was found after Ag/IgE treatment in two experiments (figure 5.10, table A2.6), and in the experiment that was performed in quadruplicate, this increase was statistically significant (figure 5.10). A significant ( $p < 0.05$ ) increase in mBMMC<sup>T-</sup> adhesion to vitronectin was also seen after treatment with A23187, but this increase was very small, and median adhesion was only 3.3% (table A2.6).

In conclusion, adhesion of mBMMC<sup>T+</sup> to laminin-1 was increased by treatment with Ag/IgE and calcium ionophore A23187. Adhesion of mBMMC<sup>T-</sup> to both fibronectin and vitronectin could be increased by treatment with Ag/IgE, but results from stimulation of adhesion of mBMMC<sup>T-</sup> to these ECM proteins with calcium ionophore were variable.

## 5.10 THE EFFECT OF DEGRANULATION OF mBMMC<sup>T+</sup> ON THE VALIDITY OF RESULTS OF ADHESION MEASURED USING THE $\beta$ -HEXOSEAMINIDASE ASSAY

### 5.10.1 *Experimental aim and design*

In 5.9.2 above, there was some variability in results of experiments measuring adhesion of Ag/IgE-treated mBMMC<sup>T+</sup> to laminin-1 using the  $\beta$ -hexoseaminidase

assay, and in preliminary experiments investigating this further (not shown), visual examination of numbers of cells adherent after Ag/IgE stimulation suggested that they were comparable to the number of cells adherent to laminin-1 after stimulation by PMA. The  $\beta$ -hexoseaminidase assay estimates cells by measurement of the amount of the lysosomal enzyme,  $\beta$ -hexoseaminidase, which is present in the lysates from adherent cells. It was hypothesised, therefore, that differences in the extent of degranulation following the two different stimuli may result in inaccuracies in quantification of adherent cells using this method. Specifically, high levels of degranulation after stimulation with Ag/IgE may result in an underestimation of the numbers of adherent cells.

An experiment was therefore set up to compare, using the  $\beta$ -hexoseaminidase assay and manual counting, adhesion of mBMMC<sup>T+</sup> to laminin-1 when stimulated by PMA, Ag/IgE or A23187. Control wells containing unstimulated cells were included; estimations using the  $\beta$ -hexoseaminidase assay were in quadruplicate, and manual counting was performed in duplicate.

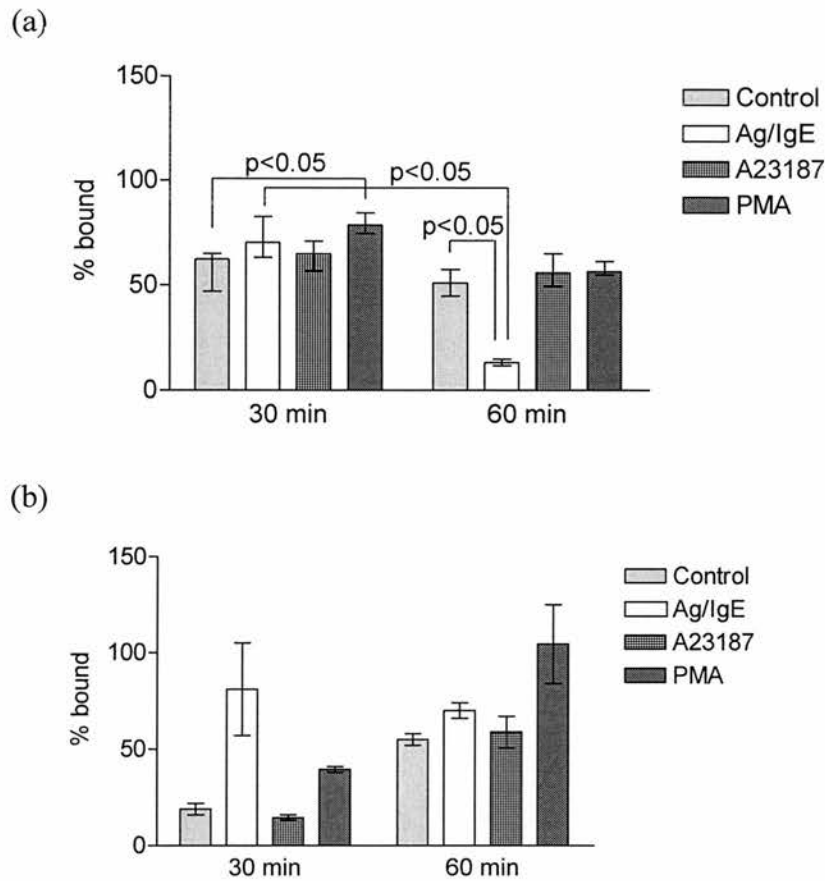
In addition to this, supernatants from wells containing adherent cells were collected for measurement of degranulation by analysis of mMCP-1 release (2.8.2). mBMMC<sup>T+</sup> contain abundant mMCP-1, and unpublished data from our laboratory showed that Ag/IgE stimulation causes release of mMCP-1 from mBMMC<sup>T+</sup>. This correlates closely with release of  $\beta$ -hexoseaminidase, which is conventionally used to measure degranulation. Therefore, as pH constraints and the presence of indicator dye in the culture medium made direct measurement of  $\beta$ -hexoseaminidase

technically infeasible, release of mMCP-1 into the culture supernatant was measured instead.

### *5.10.2 Results*

Quantitation of adhesion of mBMMC<sup>T+</sup> to laminin-1 using the  $\beta$ -hexoseaminidase assay indicated high spontaneous adhesion of mBMMC<sup>T+</sup> after 30 min and 60 min incubations (figure 5.11a). Stimulation with Ag/IgE, PMA and A23187 increased adhesion, although only PMA treatment resulted in a significant increase ( $p < 0.05$ ). As previously described in 5.9, adherent cells treated with A23187 did not flatten onto laminin-1. After 60 min, adhesion of control and PMA-stimulated cells and of cells treated with A23187 measured using the  $\beta$ -hexoseaminidase assay remained high, but there was a significant ( $p < 0.05$ ) reduction in number of cells adherent to laminin-coated wells after Ag/IgE treatment (figure 5.11a).

Visual estimation of adherent cells after 30 min and 60 min incubations did not agree with these results; high numbers of Ag/IgE-stimulated cells were observed to be adherent to laminin-coated wells after both 30 min and 60 min incubations, and after 30 min, adherent Ag/IgE stimulated cells appeared to be more abundant than PMA stimulated cells. Manual counting supported visual estimations of adherent cells. After a 30 min incubation, treatment with both Ag/IgE and PMA increased adhesion of mBMMC<sup>T+</sup> to laminin-1, but numbers adherent after Ag/IgE treatment were higher than after PMA treatment (figure 5.11b). After 60 min of incubation, increased numbers of adherent cells were counted in control, PMA or A23187 treated wells (figure 5.11b), and adhesion of Ag/IgE-treated cells remained high.



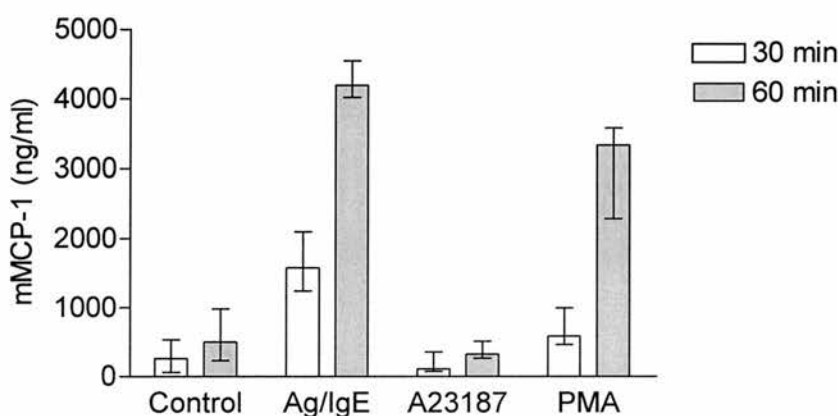
**Figure 5.11. Validation of the  $\beta$ -hexoseaminidase assay for comparison of adhesion of mBMMC<sup>T+</sup> to laminin-1 after different methods of stimulation.**

Adhesion of unstimulated (control) mBMMC<sup>T+</sup> and mBMMC<sup>T+</sup> stimulated by Ag/IgE, A23187 or PMA was analysed by (a) the  $\beta$ -hexoseaminidase assay (n=4 wells) or (b) manual counting (n=2 wells).

- The  $\beta$ -hexoaseaminidase assay indicated only a small increase in adhesion above that in controls after incubation for 30 min with Ag/IgE, A23187 or PMA. After incubation for 60 min, a significant ( $p < 0.05$ ) reduction in numbers of adherent Ag/IgE-treated cells was detected.
- Manual counting indicated that treatment with Ag/IgE and PMA increased numbers of adherent cells after 30 min. After 60 min, adhesion of cells stimulated with PMA and A23187, and of control cells had increased. Numbers of adherent Ag/IgE treated cells after 60 min was similar to those seen after 30 min.

Results are expressed as median (range) of one experiment.

Quantification of mMCP-1 in cell supernatants established that after incubation for 30 min, there was significantly ( $p<0.05$ ) higher release of mMCP-1 in Ag/IgE-treated cells than in both control and PMA stimulated cells (figure 5.12). After a 60 min incubation, mMCP-1 release from cells treated with both PMA and Ag/IgE was significantly greater than release from the same cells after 30 min, and release by control mBMMC<sup>T+</sup> after 60 min. Unexpectedly, low release was measured after treatment with A23187 (figure 5.12).



**Figure 5.12. Degranulation of mBMMC<sup>T+</sup> adherent to laminin-1 in experiment 5.10.**

Cells were stimulated with Ag/IgE, A23187 or PMA. mMCP-1 release in Ag/IgE-stimulated cells was significantly higher ( $p<0.05$ ) than in control cells after both 30 min and 60 min incubations. Also, release after 30 min was significantly higher ( $p<0.05$ ) than in PMA-stimulated cells.

mMCP-1 release in mBMMC<sup>T+</sup> stimulated by both Ag/IgE and PMA was significantly ( $p<0.05$ ) higher after 60 min than 30 min.

Results are median (range) of one experiment ( $n=4$  wells). Significant differences are omitted from the graph for clarity.



To summarise, manual estimation of adhesion of mBMMC<sup>T+</sup> to laminin-1 either spontaneously or after treatment with Ag/IgE or calcium ionophore showed stimulation of adhesion by all three mechanisms, but Ag/IgE treatment resulted in more rapid adhesion which peaked at 30 min and was maintained for 60 min (figure 5.11b). In comparison to manual estimation, the  $\beta$ -hexoseaminidase assay appeared to overestimate adhesion of unstimulated mBMMC<sup>T+</sup> and of mBMMC<sup>T+</sup> stimulated by PMA and A23187 to laminin-1 after 30 min; and underestimate adhesion of Ag/IgE treated cells relative to others at both time points (figure 5.11). These differences may, in part, be explained by the comparatively high degranulation of Ag/IgE-treated cells (figure 5.12).

## 5.11 COMPARISON OF ADHESION OF mBMMC<sup>T+</sup> FROM mMCP-1 KNOCK-OUT AND WILD-TYPE MICE TO LAMININ-1 AND FIBRONECTIN

### 5.11.1 *Experimental aim and design*

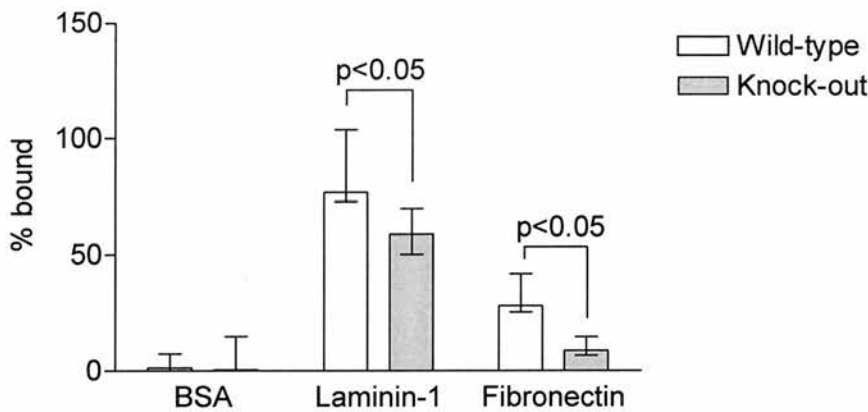
mMCP-1-expressing mBMMC<sup>T+</sup> adhered poorly to fibronectin, and unpublished data from our lab suggested fibronectin to be a possible substrate for mMCP-1, which is continually released from mBMMC<sup>T+</sup> (Miller *et al*, 1999). Therefore, it was hypothesised that adhesion of mBMMC<sup>T+</sup> to fibronectin may be attenuated by degradation of this protein by mMCP-1 released during incubation.

To test this hypothesis, adhesion of PMA-stimulated mBMMC<sup>T+</sup> from mMCP-1 knock-out and wild-type mice to fibronectin was compared in one experiment using quadruplicate wells. Adhesion to laminin-1 was also compared, and BSA-coated

wells were included as controls. PMA (50 ng/ml) was added immediately after loading of cells into coated wells and adhesion estimated after incubation for 1 h (2.11.2).

5.11.2 Results

Adhesion of mBMMC<sup>T+</sup> from mMCP-1 knock-out mice to both fibronectin and laminin-1 was significantly ( $p<0.05$ ) lower than that of mBMMC<sup>T+</sup> from wild-type mice (figure 5.13). This does not support the proposed hypothesis that degradation of fibronectin by mMCP-1 inhibits mBMMC<sup>T+</sup> adhesion to fibronectin. Adhesion of both cell-types to BSA was  $<5\%$ .



**Figure 5.13. Adhesion of mBMMC<sup>T+</sup> from mMCP-1 wild-type and knock-out mice to extracellular matrix proteins.**

Results are expressed as median (range),  $n=4$  wells.

## 5.12 DISCUSSION

Mast cells grown *in vitro* and recovered *ex vivo* have been shown to adhere to laminin, fibronectin and vitronectin (Thompson *et al*, 1989a; Dastyk *et al*, 1991; Bianchine *et al*, 1992; Columbo *et al*, 1995; Columbo & Bochner, 2001). The current study has shown that TGF- $\beta_1$  profoundly regulates adhesion of mBMMC<sup>T+</sup> (MMC homologues) to ECM proteins. The mechanism of TGF- $\beta_1$ -upregulated adhesion to laminin-1, and down-regulated adhesion to fibronectin and vitronectin may be through alteration in expression of integrins, and this is addressed in chapter 6.

TGF- $\beta_1$ -regulated expression of  $\beta_1$  integrins has been shown in several cell types (Ignotz & Massague, 1987; Heino *et al*, 1989), and in both IL-3-dependent mBMMC and mBMMC<sup>T+</sup> cultured in our lab, TGF- $\beta_1$  regulates expression of  $\alpha E$  integrin (Smith *et al*, 1994; Wright *et al*, 2002). The non-integrin LBP has also been shown to be important in adhesion of IL-3-dependent mBMMC to laminin-1, thus TGF- $\beta_1$  regulated expression of this protein might mediate adhesion of mBMMC<sup>T+</sup> to laminin-1. Mast cell adhesion to laminin and fibronectin is regulated by other mast cell growth factors including IL-3, SCF and IL-4 (Thompson *et al*, 1989a; Dastyk & Metcalfe, 1994; Lorentz *et al*, 2002). However, this upregulation was over the 60 min time period of the adhesion assay itself, and is likely to result from cytoskeletal rearrangements similar to those associated with activation by PMA (Danilov & Juliano, 1989), rather than any up or down-regulation of integrin expression.

The differential adhesion of mBMMC<sup>T+</sup> to laminin, and mBMMC<sup>T-</sup> to fibronectin and vitronectin contrasts with IL-3-dependent mBMMC, which adhere to all three ECM proteins. This may reflect different levels of differentiation of mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> in comparison to IL-3-dependent mBMMC, which are said to represent immature mast cells, but which have never been shown to have any *in vivo* counterpart. The human mast cell line, HMC-1, which may also represent an immature mast cell (Butterfield *et al*, 1988) similarly adheres to a number of ECM proteins including laminin, fibronectin, vitronectin and collagens. However, as mature human skin mast cells also adhere to laminin, fibronectin and vitronectin (Columbo *et al*, 1995; Columbo & Bochner, 2001), mast cell adhesion properties cannot be easily associated with a particular phenotype.

mBMMC<sup>T-</sup> showed variable adhesion to type I and type IV collagen (appendix 2, table A2.3). Similarly, IL-3-dependent mBMMC do not adhere to collagens, but the rat basophilic leukaemia cell line, RBL-2H3, and HMC-1 cells adhere to both type I and type IV collagen as well as to fibronectin and laminin (Hamawy *et al*, 1992; Kruger-Krasagakes *et al*, 1996).

Adhesion of IL-3-dependent mBMMC to laminins and fibronectin required activation by PMA, A23187 or FcεRI cross-linking. Our studies suggested a similar requirement for activation by mBMMC<sup>T+</sup> for adhesion to laminin-1, though high spontaneous adhesion and difficulties in enumeration of mast cells adherent after different methods of stimulation made it difficult to obtain statistically significant results. Spontaneous adhesion varied between cultures (appendix 2, tables A2.2 and

A2.4), and may have been due to low levels of endotoxin contamination, as LPS has been shown to cause mast cell degranulation (Iuvone *et al*, 1999).

Comparison of the adhesion properties of mBMMC<sup>T+</sup> stimulated by different mechanisms using the  $\beta$ -hexoseaminidase assay was not always accurate; in some experiments, visual assessment of the number of cells adherent to coated wells was not reflected by results measured using the  $\beta$ -hexoseaminidase assay. The  $\beta$ -hexoseaminidase assay produced results comparable to those achieved by manual counting when adhesion of PMA-stimulated mBMMC<sup>T+</sup> was compared to that of mBMMC<sup>T-</sup> (figure 5.8). However, when comparing adhesion of mBMMC<sup>T+</sup> stimulated by Ag/IgE, A23187, PMA or of unstimulated mBMMC<sup>T+</sup> using the  $\beta$ -hexoseaminidase assay, results were different from those obtained by manual counting (figure 5.11). In particular, the  $\beta$ -hexoseaminidase assay may underestimate numbers of Ag/IgE - stimulated cells adherent to laminin-1.

Analysis of mMCP-1 release into supernatants in order to measure degranulation of cells stimulated by the different mechanisms suggested that the comparatively high levels of degranulation of Ag/IgE-stimulated cells after 30 min and 60 min incubations may account for these underestimations, as the  $\beta$ -hexoseaminidase assay measures cell numbers based on the amount of the lysosomal enzyme,  $\beta$ -hexoseaminidase, present. Release of this enzyme from cells during degranulation may lead to an underestimation of adherent cells. The results also suggest that the  $\beta$ -hexoseaminidase assay may underestimate numbers of PMA-stimulated cells adherent, relative to control cells, due to their extensive degranulation (figure 5.12).

Surprisingly, no release was measured in cells treated with A23187, suggesting either a problem with this method of stimulation, or with the measurement of degranulation. However, as this experiment was only performed once, further repeats would be required to confirm these results. Nevertheless, this preliminary experiment assaying the release of mMCP-1 to quantify degranulation helped to explain the variability in the results of Ag/IgE-mediated adhesion of mBMMC<sup>T+</sup> to laminin-1, and supported our conclusion that adhesion of mBMMC<sup>T+</sup> to laminin-1 is stimulated by this method.

Proteases may be important for cell migration by degrading ECM components (Murphy & Gavrilovic, 1999), and unpublished work in our laboratory showed degradation of fibronectin by mMCP-1. However, comparison of adhesion of mBMMC<sup>T+</sup> from mMCP-1 knock-out and wild-type mice did not support the hypothesis that degradation by mMCP-1 might inhibit adhesion of mBMMC<sup>T+</sup> to fibronectin. Contrary to our hypothesis, significantly lower numbers of mBMMC<sup>T+</sup> from knock-out mice than from wild-type mice adhered to fibronectin and laminin-1. The reason for this is not known, and further experiments would be required to show that this was a repeatable result. Comparison of surface expression of the relevant adhesion molecules might help explain these differences.

Since mBMMC<sup>T-</sup> are phenotypically undefined mast cells, the mechanisms stimulating their adhesion to fibronectin and vitronectin were not rigorously investigated. However FcεRI cross-linking stimulated adhesion of mBMMC<sup>T-</sup> to both fibronectin and vitronectin, although variable results were obtained by

stimulation with A23187. This may have been due to technical problems, or it may be that, as the same reagent did increase adhesion of mBMMC<sup>T+</sup> to laminin-1, mBMMC<sup>T-</sup> have a higher threshold for stimulation than mBMMC<sup>T+</sup>, which adhere spontaneously to laminin-1 in some experiments. In contrast to mBMMC<sup>T-</sup>, IL-3-dependent mBMMC adhere spontaneously to vitronectin; this may reflect the different culture conditions of the two cell types, as cytokines SCF and IL-9 are also included in culture of mBMMC<sup>T-</sup>.

Epithelial basement membranes are rich in laminin, therefore if MMC *in vivo* adhere to laminin, this may support intraepithelial migration of MMC and their maintenance intraepithelially. However, although some studies (Nomizu *et al*, 1995; De Arcangelis *et al*, 1996) have reported laminin-1 to be a major component of basement membranes and located in the mouse and human small intestine (Beaulieu & Vachon, 1994; Simon-Assmann *et al*, 1994) others (Falk *et al*, 1999; Kortessmaa *et al*, 2000) reported that the isoform studied, laminin-1, is uncommon *in vivo* and is not expressed in the small intestine. This may be of relevance to the present study as some variation in affinity of laminin binding integrins for different laminin isoforms has been reported (Belkin & Stepp, 2000). The full significance will be easier to assess when the molecules mediating adhesion of mBMMC<sup>T+</sup> to laminin are identified. Low levels of adhesion of mBMMC<sup>T+</sup> to fibronectin and vitronectin, which may be expressed in the intestinal lamina propria but not basement membrane, may also reflect differentiation towards the intraepithelially located MMC phenotype driven by TGF- $\beta_1$ .

In addition to facilitating cell migration and location within tissues, adhesion of mBMMC to ECM proteins may regulate their cellular function. Adhesion of IL-3-dependent mBMMC to vitronectin enhances proliferation in response to IL-3 (Bianchine *et al*, 1992), and adhesion of HMC-1 cells to fibronectin or vitronectin provided a co-stimulatory signal for cytokine production (Kruger-Krasagakes *et al*, 1999). Also, adhesion of mBMMC to laminin-2 (Vliagoftis & Metcalfe, 1997) and of RBL-2H3 cells to fibronectin (Hamawy *et al*, 1992) enhanced secretion of mediators. Thus, mast cell adhesion to ECM proteins may act as a signal indicating their arrival at their target location within tissues, and may initiate the inflammatory response.

In conclusion, this work has shown differential adhesion of mBMMC cultured with and without TGF- $\beta_1$  to ECM proteins; further work will aim to identify the mechanisms involved.



## CHAPTER 6

### THE ROLE OF INTEGRINS AND LAMININ BINDING PROTEIN IN ADHESION OF mBMMC TO EXTRACELLULAR MATRIX PROTEINS

#### 6.1 INTRODUCTION

Results of experiments described in the previous chapter showed that TGF- $\beta_1$ -mediated differentiation of mBMMC into MMC homologues was accompanied by a down-regulation of adhesion to fibronectin and vitronectin, and upregulation of adhesion to laminin-1. Integrins mediate cell adhesion to ECM proteins (Hynes, 1992), and TGF- $\beta_1$ -regulated integrin expression has been shown in several cell types including mast cells (Ignotz & Massague, 1987; Heino *et al*, 1989; Smith *et al*, 1994; Bauvois *et al*, 1996; Rich *et al*, 1996), by mechanisms which include increased mRNA levels, increased maturation of  $\beta$  subunits and transfer of these to the plasma membrane, and increased  $\alpha\beta$  subunit assembly (Ignotz & Massague, 1987). TGF- $\beta_1$ -regulated expression of integrins might therefore account for alterations in adhesion of mBMMC to ECM proteins.

Integrins play a role in adhesion of several mast cell phenotypes to ECM proteins.  $\alpha_6\beta_1$ ,  $\alpha_4$  and  $\alpha_5\beta_1$  and  $\alpha_v\beta_3$  regulate adhesion of IL-3-dependent mBMMC and murine mast cell lines to laminin (Fehlner-Gardiner *et al*, 1996a; Vliagoftis & Metcalfe, 1997), fibronectin (Fehlner-Gardiner *et al*, 1996b) and vitronectin (Bianchine *et al*, 1992), respectively. Expression of the laminin binding integrins  $\alpha_1$  and  $\alpha_3$  by IL-3-dependent mBMMC has also been shown, although a role in

adhesion has been identified for neither of these integrins (Vliagoftis & Metcalfe, 1997). Adhesion of mBMMC<sup>T-</sup> to fibronectin and vitronectin, and of mBMMC<sup>T+</sup> to laminin is likely to depend on similar integrins, expression of which could be regulated by TGF- $\beta_1$ . Additionally, the non-integrin laminin binding protein (LBP) has been shown to participate in adhesion of IL-3-dependent mBMMC to laminin-1 (Thompson *et al*, 1989a), and may also regulate adhesion of mBMMC<sup>T+</sup> to this protein.

The aim of the following experiments was to investigate the hypothesis that TGF- $\beta_1$  regulates adhesion of mBMMC to ECM proteins via alteration of expression of adhesion molecules, including integrins and LBP. To this end, expression of several integrins and LBP has been compared in mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup>, and their role in adhesion to ECM proteins investigated using neutralising antibodies. In addition to integrins previously implicated in mast cell adhesion, the role of the laminin binding integrin,  $\alpha 7$  in adhesion of mBMMC<sup>T+</sup> has been investigated. This integrin was originally thought to be skeletal muscle-specific (von der Mark *et al*, 1991), but expression of the  $\alpha 7B$  isoform has since been found in other tissues, including intestinal epithelium, where it correlates with intestinal cell differentiation (Basora *et al*, 1997).

## 6.2 ANALYSIS OF EXPRESSION OF ADHESION MOLECULES BY mBMMC<sup>T-</sup> AND mBMMC<sup>T+</sup> USING SEMI-QUANTITATIVE RT-PCR

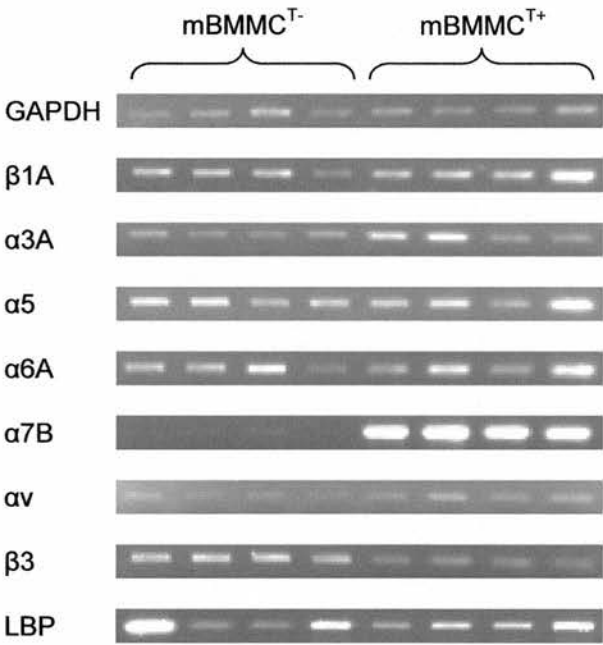
### 6.2.1 *Experimental aim and design*

Alteration of the rate of transcription or in the stability of mRNA is one possible mechanism of regulation of adhesion molecule expression by TGF- $\beta_1$  (Ignotz & Massague, 1987). Therefore, as published primer sequences are available for many of the integrins previously implicated in mast cell adhesion to ECM proteins, expression of adhesion molecules by mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> was first examined by RT-PCR. Expression of integrin subunits  $\beta_1$ ,  $\alpha_3$ ,  $\alpha_5$ ,  $\alpha_6$ ,  $\alpha_v$  and  $\beta_3$ , which has been previously described in murine IL-3-dependent BMMC (Bianchine *et al*, 1992; Fehlner-Gardiner *et al*, 1996a; Fehlner-Gardiner *et al*, 1996b; Vliagoftis & Metcalfe, 1997), was analysed. Additionally, expression of the non-integrin LBP, and the laminin binding integrin  $\alpha_7$  was investigated.

Expression of adhesion molecules was analysed in mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> from 4 separate cultures. Mast cells used for all studies in this chapter were generally between 14 and 30 days old (2.4.1). mBMMC were collected into TRI reagent (2.4.10), and expression of adhesion molecules analysed by RT-PCR (2.5.1 - 8) with each primer being optimised for semi-quantitative analysis (2.5.8). Specific conditions for each primer are shown in table 2.3.

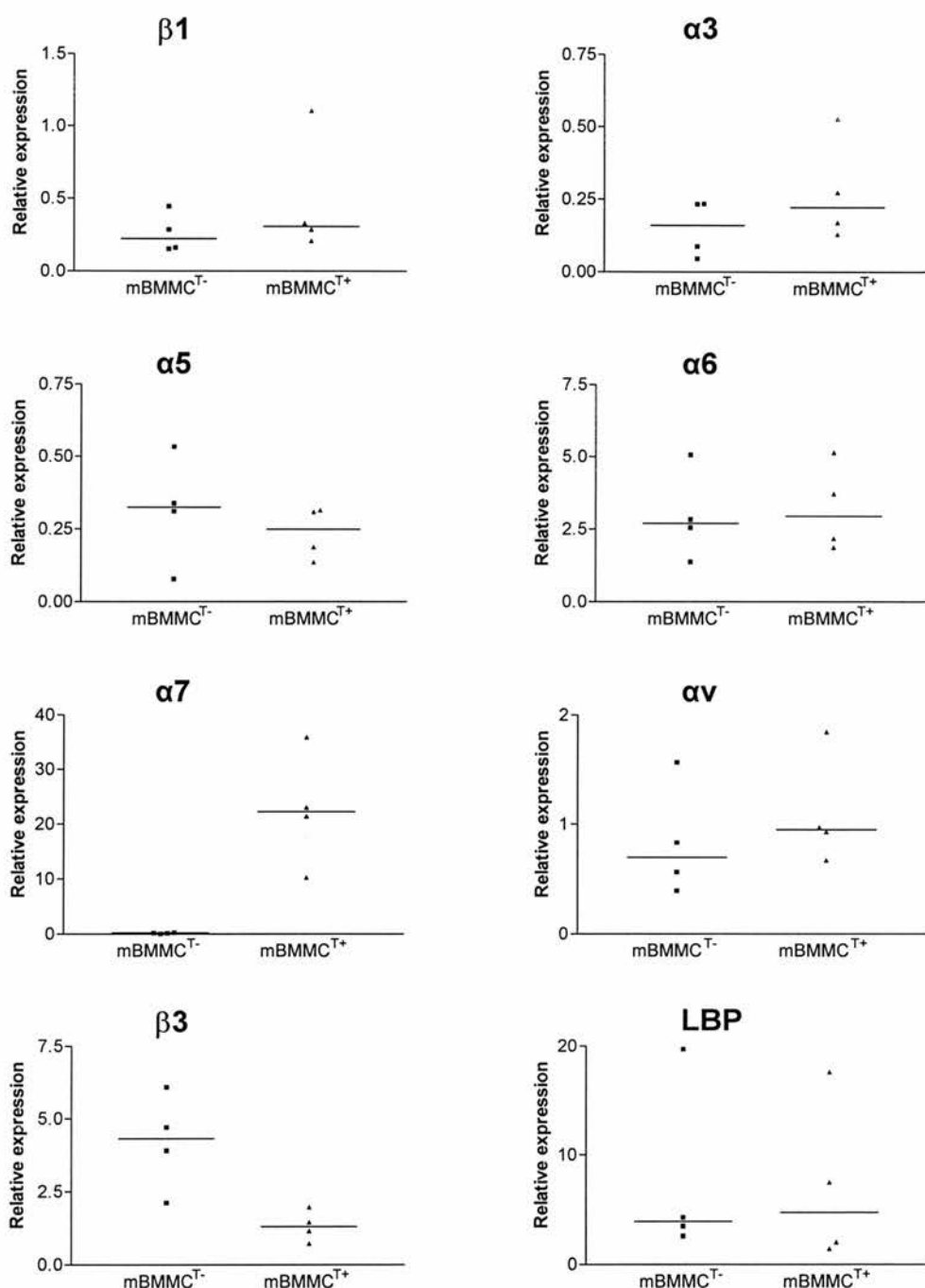
6.2.2 Results

RT-PCR analysis of adhesion molecule expression by mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> showed a consistent and substantial upregulation of  $\alpha 7$  transcripts in the presence of TGF- $\beta_1$  (figure 6.1). Expression of  $\alpha 7$  integrin was low-to-undetectable after 35 cycles of PCR in mBMMC<sup>T-</sup>, whereas inclusion of 1 ng/ml TGF- $\beta_1$  in cultures resulted in a significant ( $p<0.05$ ) increase in  $\alpha 7$ B mRNA expression by mBMMC<sup>T+</sup> (figures 6.1 and 6.2). The  $\alpha 7$  primers could amplify both  $\alpha 7$ A and  $\alpha 7$ B mRNA resulting in PCR products of 480 BP and 366 BP respectively (table 2.3), but only a single band of 366 BP was detected, indicating expression of  $\alpha 7$ B transcripts only.



**Figure 6.1. Analysis of adhesion molecule expression in mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup>.**

Expression was analysed in 4 separate cultures of each cell type by semi-quantitative RT-PCR.  $\alpha 7$ B integrin mRNA was highly upregulated in mBMMC cultured with TGF- $\beta_1$ , whereas TGF- $\beta_1$  moderately down-regulated expression of integrin  $\beta 3$ . Transcripts for other adhesion molecules were expressed at comparable levels by both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup>.



**Figure 6.2. Relative expression of integrin subunits and LBP by mBMMC<sup>-</sup> and mBMMC<sup>+</sup>.**

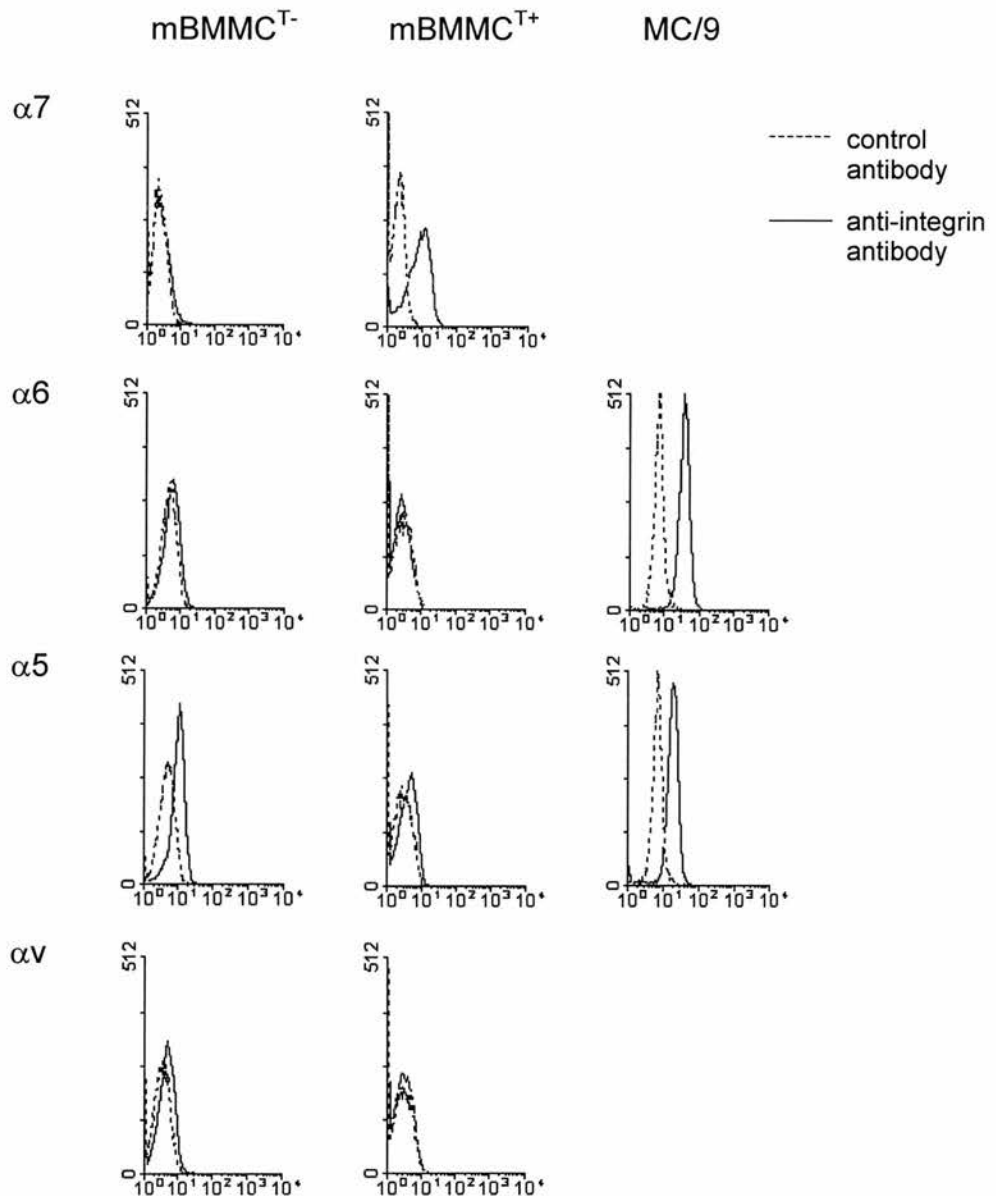
Expression was analysed in cells from 4 separate cultures by semi-quantitative RT-PCR. Expression of α7B integrin was significantly ( $p < 0.05$ ) higher in mBMMC<sup>+</sup> than mBMMC<sup>-</sup>. Also, expression of β3 integrin was significantly ( $p < 0.05$ ) higher in mBMMC<sup>-</sup> than mBMMC<sup>+</sup>.

In contrast, expression of  $\beta 3$  mRNA, whilst being detected in both cell types, was significantly down-regulated by TGF- $\beta_1$  ( $p < 0.05$ ) (figures 6.1 and 6.2). Expression of  $\beta 1A$ ,  $\alpha 3A$ ,  $\alpha 5$ ,  $\alpha 6A$ ,  $\alpha v$  and LBP was detected at comparable levels in both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup>.

### 6.3 FLOW CYTOMETRIC ANALYSIS OF SURFACE EXPRESSION OF INTEGRINS BY mBMMC<sup>T-</sup> AND mBMMC<sup>T+</sup>

#### 6.3.1 *Experimental aim and design*

RT-PCR analysis showed expression of mRNA for the laminin binding integrin  $\alpha 7B$  in mBMMC<sup>T+</sup>, and down-regulated expression of  $\beta 3$  integrin, compared to levels in mBMMC<sup>T-</sup>. Since the main focus of this work was on mBMMC<sup>T+</sup>, as potential MMC homologues, flow cytometry was used to confirm that TGF- $\beta_1$ -mediated upregulation of  $\alpha 7B$  mRNA resulted in surface expression of  $\alpha 7$  integrin. Also, in the absence of differences at the mRNA level, and as surface expression of these integrins by IL-3-dependent mBMMC and a role in adhesion to ECM proteins has previously been reported, expression of  $\alpha 5$ ,  $\alpha 6$  and  $\alpha v$  by mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> from 3 separate cultures was analysed by flow cytometry (2.12 and 2.13). In addition, a murine mast cell line, MC/9 (2.4.3), which has been shown previously to express integrin  $\alpha 5$  (Fehlner-Gardiner *et al*, 1996b) and  $\alpha 6$  (Fehlner-Gardiner *et al*, 1996a) was used as a positive control for expression of these integrins.



**Figure 6.3. Surface expression of integrins in mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup>.**

MC/9 cells were included as positive controls for expression of α5 and α6 integrins.

mBMMC<sup>T+</sup> expressed α7 integrin, whereas no expression was detected in mBMMC<sup>T-</sup> cultured without TGF-β<sub>1</sub>.

Expression of α6 was virtually absent in both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup>; high expression was detected in the positive control MC/9 cells.

α5 integrin was expressed by mBMMC<sup>T-</sup> but there was decreased expression in mBMMC<sup>T+</sup>, and high expression in MC/9 cells.

Expression of integrin αv was virtually absent in both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup>. These flow cytometry experiments were repeated twice more using cells from separate cultures, with similar results.

### 6.3.2 Results

Surface expression of  $\alpha 7$  was consistently detected in TGF- $\beta_1$ -supplemented mBMMC<sup>T+</sup>, whereas no expression was detected in mBMMC<sup>T-</sup> (figure 6.3). In contrast, surface expression of  $\alpha 5$  was increased in mBMMC<sup>T-</sup> compared to mBMMC<sup>T+</sup>, but expression in both mBMMC phenotypes was lower than seen in the positive control MC/9 cells. Expression of  $\alpha 6$  was virtually absent in mBMMC<sup>T+</sup> and mBMMC<sup>T-</sup> but, as expected, was high in MC/9 cells (figure 6.3). Expression of  $\alpha v$  was low-to-absent in both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup>.

## 6.4 ANALYSIS OF EXPRESSION OF LAMININ BINDING PROTEIN BY mBMMC<sup>T-</sup> AND mBMMC<sup>T+</sup> USING WESTERN BLOTTING

### 6.4.1 Experimental aim and design

mRNA for LBP was detected at similar levels in both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup>, but LBP is reported to be post-transcriptionally regulated (Landowski *et al*, 1995) so mRNA levels might not reflect cellular protein levels, therefore expression of LBP in mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> was further investigated by Western blotting.

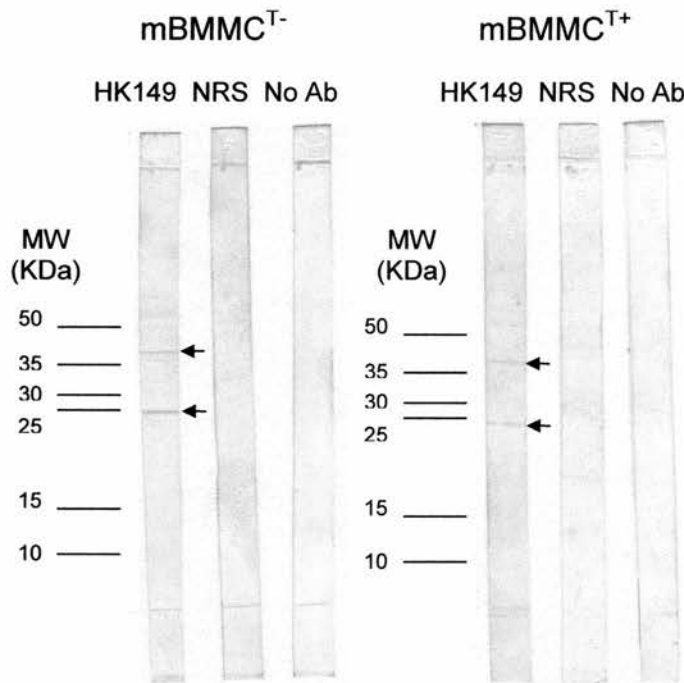
Protein was extracted from one sample of mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> (2.9.1.)

Samples were then electrophoresed on separate Laemmli gels (2.9.2.1), transferred onto membranes (2.9.4), and immunodetection performed using rabbit polyclonal antiserum raised against the N-terminal region of LBP (2.9.7).



### 6.4.2 Results

Bands reactive with the anti-LBP polyclonal serum HK149 were detected on Western blots at approximately 25 kDa and 37 kDa in proteins separated from mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> (figure 6.4). These were lower values than the published 32 and 45 kDa molecular weights (Clement *et al*, 1990). Blots exposed to normal rabbit serum or incubated in the absence of primary antibody were negative (figure 6.4).



**Figure 6.4. Western blots of laminin binding protein (LBP) in mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup>.**

To detect LBP, HK149 polyclonal serum raised against a 17-mer synthetic peptide from the NH<sub>2</sub>-terminal region of LBP-32 (residues 25 – 41) was used. The 25 and 37 kDa proteins detected (arrowed) were of lower molecular weight than the 32 and 45 kDa LBP bands reported to be recognised by this antibody.

Control blots were incubated with normal rabbit serum (NRS) or blocking buffer only, with no antibody (no Ab); and were negative.

## 6.5 USE OF NEUTRALISING ANTIBODIES TO INVESTIGATE THE ROLE OF INTEGRINS IN ADHESION OF mBMMC TO ECM PROTEINS

### 6.5.1 *Experimental aim and design*

Experiment 6.4 demonstrated expression of  $\alpha 7$  integrin by mBMMC<sup>T+</sup> only, and that there was higher surface expression of  $\alpha 5$  integrin by mBMMC<sup>T-</sup> than by mBMMC<sup>T+</sup>. We therefore wished to determine if expression of these integrins accounted for the differences in adhesion to laminin and fibronectin in these cells. Also, whereas previous studies (Fehlner-Gardiner *et al*, 1996a; Vliagoftis & Metcalfe, 1997) showed expression of  $\alpha 6$  integrin by IL-3-dependent mBMMC and adhesion of these cells to laminin-1 was inhibited by preincubation with the  $\alpha 6$ -neutralising antibody GoH3, expression of  $\alpha 6$  was low in both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup>. We were therefore interested to know what role, if any, this integrin had to play in adhesion of mBMMC<sup>T+</sup> to laminin-1. Use of the anti- $\alpha v$  monoclonal RMV-7 in flow cytometry showed low-to-absent expression in both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup>, but as antibodies to  $\alpha v \beta 3$  have previously been shown to block adhesion of IL-3-dependent mBMMC to vitronectin, the effect of RMV-7 on mBMMC<sup>T-</sup> adhesion to vitronectin was also investigated.

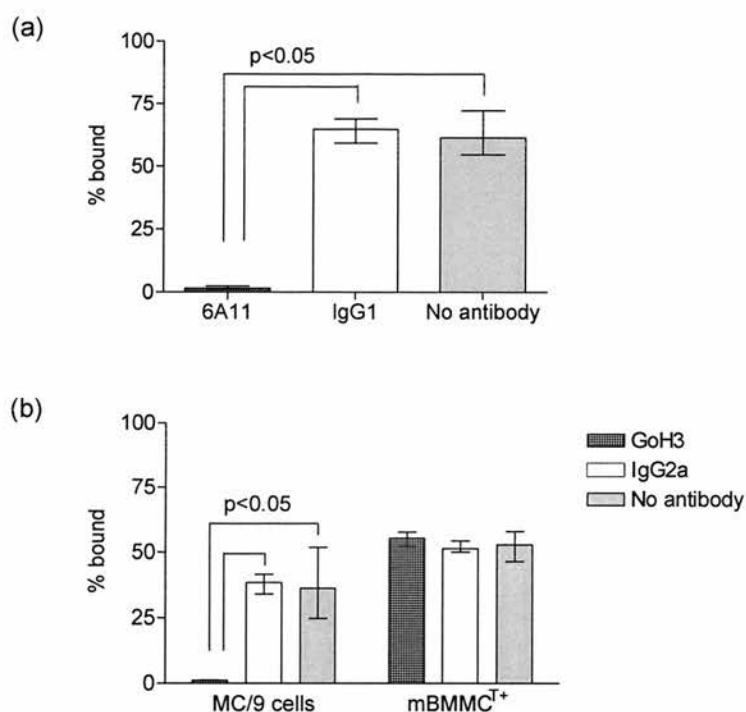
In order to investigate the role of  $\alpha 6$  and  $\alpha 7$  integrins in the adhesion of mBMMC<sup>T+</sup> to laminin-1, and of  $\alpha 5$  and  $\alpha v$  integrins in the adhesion of mBMMC<sup>T-</sup> to fibronectin and vitronectin, respectively, cells were preincubated with appropriate antibodies (2.15) before pipetting into laminin, fibronectin or vitronectin-coated wells (2.11.1).

PMA (50 ng/ml) was then immediately added, and the number of adherent cells after incubation for 1 h measured (2.11.2).

As others (Thompson *et al*, 1989a; Thompson *et al*, 1990) showed a role for LBP in adhesion of IL-3-dependent mBMMC to laminin-1, the role of LBP in adhesion of mBMMC<sup>T+</sup> was also investigated in preliminary experiments, by preincubation with either rabbit anti-LBP polyclonal serum (HK48, raised against a LBP fusion protein as described in (Clement *et al*, 1990), kindly provided by Hynda Kleinmann, National Institute of Dental Research, N.I.H., Bethesda, U.S.A.) or normal rabbit serum. However, these attempts were unsuccessful due to background inhibition of adhesion by normal rabbit serum, and this line of investigation was not pursued.

### 6.5.2 Results

Use of the neutralising antibody, 6A11, clearly demonstrated a role for  $\alpha 7$  integrin in adhesion of mBMMC<sup>T+</sup> to laminin-1. Preincubation with 6A11 at 10  $\mu$ g/ml resulted in a 98% reduction in adhesion of PMA-stimulated mBMMC<sup>T+</sup> to laminin-1 (Figure 6.5a), which was statistically significant ( $p < 0.05$ ). This experiment was repeated twice more in quadruplicate and triplicate, with a reduction in adhesion of 99.8% and 100% (appendix 2, table A2.7). In contrast, use of the anti- $\alpha 6$  monoclonal GoH3 (1  $\mu$ g/ml) had no effect on adhesion of mBMMC<sup>T+</sup> to laminin-1 (figure 6.5b; appendix 2, table A2.8), whereas adhesion of MC/9 cells, which are reported to express the integrin  $\alpha 6\beta 1$  (Fehlner-Gardiner *et al*, 1996a) and were used as a positive control for this antibody, was reduced by 98% in the presence of GoH3 (figure 6.5b).

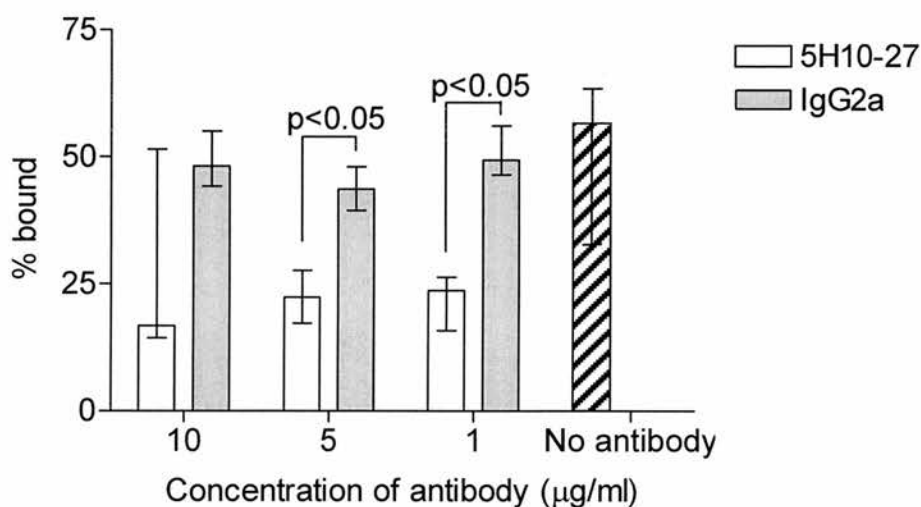


**Figure 6.5. Antibody blocking of the adhesion of mBMMC<sup>T+</sup> to laminin-1.**

- (a) The  $\alpha 7$  integrin-neutralising antibody 6A11 (10  $\mu\text{g/ml}$ ) significantly reduced adhesion of PMA-stimulated mBMMC<sup>T+</sup> to laminin-1 by 98% ( $p < 0.05$ ).
- (b) In contrast, the  $\alpha 6$ -neutralising antibody GoH3 (1  $\mu\text{g/ml}$ ) had no effect on adhesion of mBMMC<sup>T+</sup>, but significantly ( $p < 0.05$ ) reduced adhesion of MC/9 cells to laminin-1 by 98%.

Results are median (range) ( $n=4$  wells) for single experiments which were repeated twice with similar results (appendix 2, table A2.7 and A2.8).

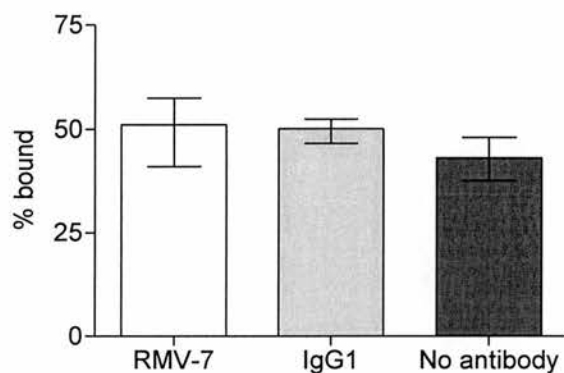
Preincubation of mBMMC<sup>T-</sup> with the anti- $\alpha 5$  monoclonal antibody 5H10-27 resulted in a partial inhibition of adhesion to fibronectin. In a quadruplicate experiment where mBMMC<sup>T-</sup> were pre-incubated with 5H10-27 at 1, 5 or 10  $\mu\text{g/ml}$ , statistically significant reductions in adhesion to fibronectin of 52.0% and 48.7% were obtained at 1 and 5  $\mu\text{g/ml}$ , and a 65.0% reduction was obtained by preincubation at 10  $\mu\text{g/ml}$ , though this was not statistically significant (figure 6.6; appendix 2, table A2.9).



**Figure 6.6. Antibody blocking of the adhesion of mBMMC<sup>T-</sup> to fibronectin.**

Effect of the anti- $\alpha 5$  integrin blocking antibody, 5H10-27 or IgG2a control on adhesion of mBMMC<sup>T-</sup> to fibronectin (20  $\mu$ g/ml).

5H10-27 resulted in a significant ( $p < 0.05$ ) reduction in adhesion when used at 1 or 5  $\mu$ g/ml. Results are shown as median (range) of one experiment,  $n = 4$  wells. Repeat experiments are shown in appendix 2, table A2.9.



**Figure 6.7. Failure of antibody to block the adhesion of mBMMC<sup>T-</sup> to vitronectin.**

Effect of anti- $\alpha v$  monoclonal RMV-7 or IgG1 control (10  $\mu$ g/ml) on adhesion of mBMMC<sup>T-</sup> to vitronectin (10  $\mu$ g/ml).

Preincubation with RMV-7 did not significantly inhibit adhesion of mBMMC<sup>T-</sup> to vitronectin. Results are shown as median (range) of one experiment ( $n = 4$  wells).

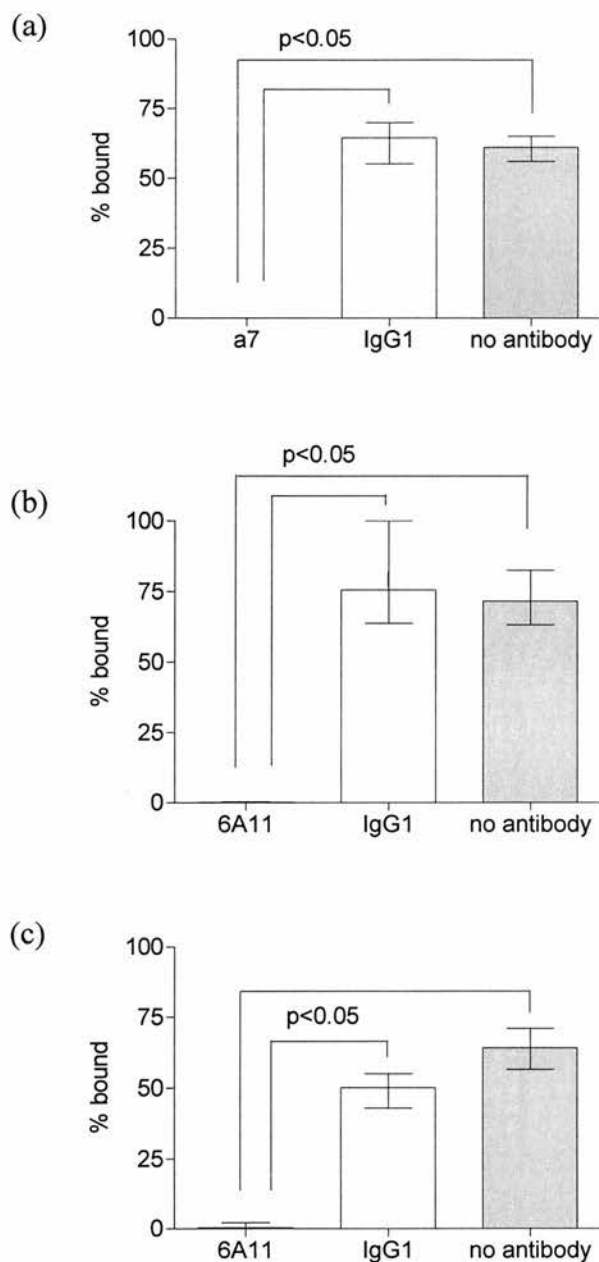
However, results of repeat experiments, where cells were preincubated with 5H10-27 or isotype control at 10  $\mu\text{g/ml}$ , were more variable (appendix 2, table A2.9), though statistically significant reductions in adhesion of mBMMC<sup>T+</sup> to fibronectin of 19.0% and 26.8% were obtained. Preincubation of mBMMC<sup>T+</sup> with the anti- $\alpha 7$  monoclonal RMV-7 had no effect on adhesion to vitronectin (figure 6.7).

## 6.6 USE OF NEUTRALISING ANTIBODIES TO INVESTIGATE THE ROLE OF $\alpha 7$ INTEGRIN IN SPONTANEOUS, Ag/IgE-STIMULATED AND CALCIUM IONOPHORE A23187-STIMULATED ADHESION OF mBMMC<sup>T+</sup> TO LAMININ-1.

### 6.6.1 *Experimental aim and design*

The  $\alpha 7$ -neutralising antibody, 6A11 blocked adhesion of PMA-stimulated mBMMC<sup>T+</sup> to laminin-1. However, mBMMC<sup>T+</sup> adhere spontaneously to laminin-1, and adhesion is increased by treatment with Ag/IgE or calcium ionophore A23187. This experiment aimed to investigate if adhesion via these mechanisms was also mediated by  $\alpha 7$  integrin, and could therefore be blocked by 6A11.

To investigate the role of  $\alpha 7$  in spontaneous and A23187-stimulated adhesion of mBMMC<sup>T+</sup> to laminin-1, mBMMC<sup>T+</sup> were preincubated with 6A11 as described in 6.5, before loading into quadruplicate wells and addition of no stimulating agent, or 1  $\mu\text{M}$  A23187. For investigation of the role of  $\alpha 7$  in adhesion of Ag/IgE-stimulated mBMMC<sup>T+</sup> to laminin-1, mBMMC<sup>T+</sup>, sensitised by overnight incubation with IgE anti-DNP (2.11.3), were preincubated with 6A11 before loading into quadruplicate



**Figure 6.8. Antibody blocking of the adhesion of mBMMC<sup>T+</sup> to laminin after stimulation by different mechanisms.**

The effect of the  $\alpha 7$  integrin-neutralising antibody 6A11 (10  $\mu$ g/ml) on (a) spontaneous, (b) Ag/IgE-stimulated and (c) calcium ionophore A23187-stimulated adhesion of mBMMC<sup>T+</sup> to laminin. 6A11 almost completely blocks adhesion stimulated by all 3 mechanisms.

Results are expressed as median (range) of one experiment (n=4 wells).

laminin-coated (2.11.1) wells and addition of DNP-HSA (2.11.3). The number of mBMMC<sup>T+</sup> adherent after incubation for 1 h was then analysed (2.11.2).

### 6.6.2 Results

Spontaneous, Ag/IgE-stimulated and A23187-stimulated adhesion of mBMMC<sup>T+</sup> to laminin-1 was almost completely blocked by incubation with 6A11, but not control IgG1 (figure 6.8). This shows that  $\alpha 7$  integrin also mediates adhesion of mBMMC<sup>T+</sup> stimulated by these mechanisms.

## 6.7 EXPRESSION OF $\alpha 7$ INTEGRIN IN MURINE JEJUNAL EPITHELIUM

### 6.7.1 Experimental aim and design

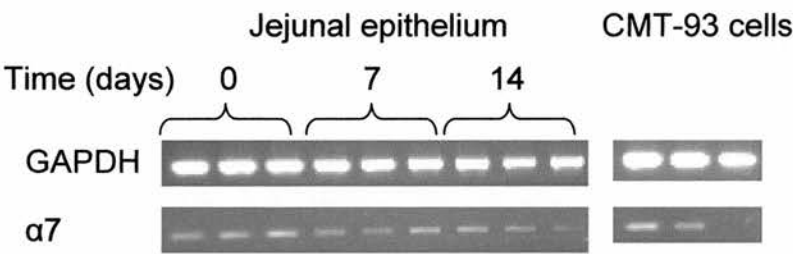
Surface expression of  $\alpha 7$  integrin has been shown in mBMMC<sup>T+</sup>, and a role for this integrin in mBMMC<sup>T+</sup> adhesion to laminin-1 demonstrated, but it is not known if this integrin is expressed by intraepithelial MMC *in vivo*. Expression of mMCP-1 in intestinal epithelium was detected on days 7 and 14 post *N. brasiliensis* infection (3.2.2.2), coinciding with increased numbers of intraepithelial mast cells. Therefore, if expression of  $\alpha 7$  integrin by MMC was high in comparison to background epithelial expression, increased expression of  $\alpha 7$  mRNA might also be detected in intestinal epithelium on days 7 and 14 post-infection.

Expression of  $\alpha 7$  integrin in control (uninfected) murine jejunal epithelium, and in jejunal epithelium collected on days 7 and 14 post-infection (experiment 3.2) was therefore analysed by RT-PCR (6.2). Additionally, expression of  $\alpha 7$  was analysed by RT-PCR in 3 samples of CMT-93 cells (2.4.4).



6.7.2 Results

Using RT-PCR, expression of  $\alpha 7$ B integrin was detected in both uninfected and day 7 and 14 infected epithelial samples (figure 6.9). Although semi-quantitative analysis was not performed,  $\alpha 7$ B integrin appeared to be expressed at similar levels in all jejunal epithelial samples. Expression of  $\alpha 7$ B was also detected in 2 out of 3 samples of CMT-93 epithelial cells, suggesting that epithelial cells may be a constitutive source of  $\alpha 7$  expression in jejunal epithelium.



**Figure 6.9. Expression of  $\alpha 7$ B integrin in jejunal epithelium.**

Expression was analysed in murine jejunal epithelium collected 0 (uninfected control), 7 and 14 days post-infection with *N. brasiliensis*; and in CMT-93 epithelial cells.

$\alpha 7$  was expressed in both control and infected jejunal epithelium, and expression was detected in 2 out of 3 samples of CMT-93 epithelial cells.

6.8 EXPRESSION OF EXTRACELLULAR SPLICE VARIANTS OF  $\alpha 7$   
INTEGRIN BY mBMMC<sup>T+</sup>

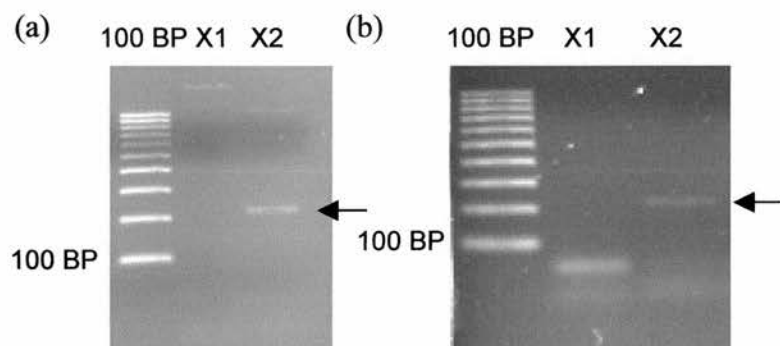
6.8.1 Experimental aim and design

Isoforms of  $\alpha 7$  integrin,  $\alpha 7$ A and  $\alpha 7$ B, result from alternative splicing of an intracellular region of  $\alpha 7$ , and expression of  $\alpha 7$ B was shown in mBMMC<sup>T+</sup> by RT-PCR. However, two extracellular splice variants of  $\alpha 7$ , named  $\alpha 7$ X1 and  $\alpha 7$ X2,

have also been described (Ziober *et al*, 1993), and cells expressing either isoform adhere differentially to isoforms of laminin (von der Mark *et al*, 2002). It was therefore important to identify the extracellular splice variants of  $\alpha 7$  that are expressed by mBMMC<sup>T+</sup>. To this end, expression of  $\alpha 7$ X1 and X2 in mBMMC<sup>T+</sup> was investigated by RT-PCR (2.5.1 – 7) in one sample of mBMMC<sup>T+</sup> RNA extracted for experiment 6.2. Specific conditions and primers used are detailed in table 2.4. As a positive control, expression was also analysed in murine cardiac muscle, which is reported to express both  $\alpha 7$ X1 and  $\alpha 7$ X2 (Ziober *et al*, 1997). Murine heart was collected into RNA-later<sup>TM</sup> and RNA extracted as described for jejunum in 2.3.4.

### 6.8.2 Results

The X2 isoform of  $\alpha 7$  was expressed in both heart and mBMMC<sup>T+</sup> samples (figure 6.10). However, expression of the X1 isoform could be detected in neither sample, even after thorough optimisation of the PCR protocol, suggesting a problem with the primers.



**Figure 6.10. Expression of extracellular splice variants of  $\alpha 7$ , X1 and X2.**

Expression was analysed in (a) mBMMC<sup>T+</sup> and (b) adult murine heart. Primers are reported to amplify 200 BP and 220 BP products, corresponding to the X1 and X2 domains of  $\alpha 7$ .

Only expression of X2 was detected in mBMMC<sup>T+</sup> and murine adult heart, which is reported to express both X1 and X2 isoforms. Arrows indicate bands representative of X2 expression in mBMMC<sup>T+</sup> and heart.

## 6.9 DISCUSSION

The previous chapter showed TGF- $\beta_1$ -regulated adhesion of mBMMC to laminin-1, and this chapter has revealed the mechanism as being upregulation of expression of integrin  $\alpha 7$ . Additionally, supplementation with TGF- $\beta_1$  was associated with down-regulation of  $\alpha 5$  and  $\beta 3$ , and this may contribute towards reduced adhesion of mBMMC<sup>T+</sup> to fibronectin and vitronectin.

$\alpha 7\beta 1$  has been described as being muscle-specific (Belkin & Stepp, 2000) and, though several non-muscle locations have been described (Velling *et al*, 1996; Klaffky *et al*, 2001), expression has not previously been shown in leucocytes of any subset. Importantly, TGF- $\beta_1$ -regulated expression of  $\alpha 7$  has not been shown in any cell type, and though developmental regulation of  $\alpha 7$  has been described in muscle and proposed for intestinal epithelium (Basora *et al*, 1997), the molecular signals controlling expression in these cells are unknown.

The  $\alpha 7B$  isoform expressed by mBMMC<sup>T+</sup> is also expressed in skeletal myoblasts, the  $\alpha 7A$  isoform being restricted exclusively to mature skeletal muscle (Collo *et al*, 1993; Ziober *et al*, 1997; Schöber *et al*, 2000). However, as both isoforms promote adhesion on laminin-1 and laminin-2/4 (Yao *et al*, 1996; Echtermeyer *et al*, 1996), which may be rich in epithelial basement membranes (Simon-Assmann *et al*, 1994),  $\alpha 7B$  expression *in vivo* could either promote intraepithelial migration or limit egress of MMC from the epithelium into the lamina propria via adhesion to BM laminin. Additionally, restricted expression of  $\alpha 7B$  by epithelial cells of the crypt-villous junction (Basora *et al*, 1997), the primary location of intraepithelial MMC *in vivo*

(Friend *et al*, 1996), suggests  $\alpha 7$  may be important for adhesion of both epithelial cells and mast cells to the basement membrane at this site.

To investigate if  $\alpha 7$  integrin could be expressed by intraepithelial MMC *in vivo*, expression was analysed in jejunal epithelium from uninfected and *N. brasiliensis* infected mice. If background expression was sufficiently low, increased levels of expression caused by intraepithelial migration of MMC might be detected on days 7 and 14 post-infection. However, expression of  $\alpha 7B$  mRNA was detected in jejunal epithelium from both uninfected and infected mice (figure 6.9), and appeared to be at similar levels, though semi-quantitative analysis was not performed. This is in agreement with previous work showing expression of  $\alpha 7B$  transcripts in murine small intestine (Collo *et al*, 1993). CMT-93 epithelial cells also expressed  $\alpha 7B$  (figure 6.9), suggesting that jejunal epithelial cells, similarly, may be the main source of  $\alpha 7$ . If  $\alpha 7$  is expressed by intraepithelial MMC, it is not at sufficiently high levels to substantially increase expression in jejunal epithelium.

The extracellular splice variant of  $\alpha 7$  which is expressed by  $mBMMC^{T+}$  was also investigated, as differential adhesion to isoforms of laminin have been shown in cells expressing either  $\alpha 7$  X1 or X2 (von der Mark *et al*, 2002). Both X1 and X2 promote adhesion to laminin-2, but X1 shows high affinity binding to laminins-8 and -10/11, whereas X2 binds to laminin-1 with much higher affinity than X1. Expression of X2 by  $mBMMC^{T+}$  is therefore consistent with their adhesion to laminin-1; expression of X1 was detected in neither  $mBMMC^{T+}$  nor murine heart, which was used as a

positive control (figure 6.10), therefore it is unclear if this isoform is expressed by mast cells.

Previous studies (Fehlner-Gardiner *et al*, 1996a; Vliagoftis & Metcalfe, 1997) have implicated integrin  $\alpha 6$  in promotion of murine mast cell adhesion to laminin-1 and -2, however these studies used several mast cell lines and IL-3-dependent mBMMC, which may be more representative of immature mast cells. mMCP-1<sup>+</sup> mBMMC<sup>T+</sup> closely resemble intraepithelial MMC (Scudamore *et al*, 1997; Miller *et al*, 1999), and whilst these cells expressed  $\alpha 6$  transcripts at levels similar to those of mBMMC<sup>T-</sup> (figures 6.1 and 6.2) flow cytometry showed low expression of  $\alpha 6$  in both cell types (figure 6.3). This made  $\alpha 6$  an unlikely candidate for regulation of mBMMC<sup>T+</sup> adhesion to laminin, and inclusion of the anti- $\alpha 6$  monoclonal GoH3 in adhesion assays had no effect on adhesion of mBMMC<sup>T+</sup> to laminin-1 (figure 6.5). Positive expression of  $\alpha 6$  by flow cytometry in MC/9 cells, and GoH3-mediated inhibition of adhesion to laminin-1 further supports our conclusion that  $\alpha 6$  integrin plays no role in adhesion of mBMMC<sup>T+</sup> to laminin-1.

Human skin mast cells also adhere to laminin, but do not significantly express  $\alpha 6$  integrin, and adhesion is inhibited by antibodies to  $\alpha 3$  integrin (Columbo *et al*, 1995). Antibodies to  $\alpha 3$  integrin were not included in our studies, but in view of conclusive evidence for the role of  $\alpha 7$  integrin in adhesion of mBMMC<sup>T+</sup> (MMC homologues), and because the expression of  $\alpha 3$  mRNA expression in mBMMC<sup>T+</sup> was similar to that in mBMMC<sup>T-</sup> which do not adhere to laminin, a major role for  $\alpha 3$

integrin in laminin binding seems unlikely in MMC. The importance of different integrins in adhesion to laminin may vary between mast cell phenotypes,  $\alpha 6$  possibly playing a role only in adhesion of immature mast cells, whilst  $\alpha 3$  and  $\alpha 7$  may mediate adhesion of mature connective tissue mast cells and MMC, respectively to laminin.

LBP has been implicated in adhesion of IL-3-dependent mBMMC to laminin-1 (Thompson *et al*, 1989a; Thompson *et al*, 1990), but expression of LBP mRNA was similar in both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup>. Also, 37 and 25 kDa bands, which may have represented LBP, were detected by Western blotting in both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> (figure 6.4). The antibody used, which was raised against a 17-mer synthetic peptide from the N-terminal region of LBP 32, has been reported to recognise 32 and 45 kDa proteins (Clement *et al*, 1990), but detection of proteins running slightly lower at 42 and 31 kDa has also been reported (Nomizu *et al*, 1995). The bands detected may represent expression of LBP but, as expression of LBP is post-transcriptionally regulated (Landowski *et al*, 1995) and both intracellular and cell-surface forms exist, detection of protein expression does not necessarily reflect surface expression of receptor.

Functional studies would be required to demonstrate a role for LBP in adhesion of mBMMC<sup>T+</sup> to laminin-1, as in IL-3-dependent mBMMC (Thompson *et al*, 1989a; Thompson *et al*, 1990), but attempts were unsuccessful due to high background inhibition of adhesion using control serum. Studies of T-lymphocytes (Canfield & Khakoo, 1999) showed that  $\alpha 6$ -mediated adhesion to laminin is dependent on co-

expression of LBP suggesting possible co-ordinate interactions between integrins and LBP. Therefore, a role for LBP in adhesion of mBMMC<sup>T+</sup> to laminin-1 cannot be excluded, as LBP may also act as a co-receptor for integrin  $\alpha 7$ .

$\alpha 5$  integrin is expressed throughout differentiation of both IL-3-dependent mBMMC and human CBMC (Tachimoto *et al*, 2001), and is also expressed by mature human tissue mast cells in lung, skin and uterus (Sperr *et al*, 1992), but expression of this integrin appears not to be a feature of mBMMC<sup>T+</sup>. As shown by flow cytometry (figure 6.3), functional expression of  $\alpha 5$  in mBMMC may be post-transcriptionally down-regulated since mRNA levels of this integrin were similar in both mBMMC<sup>T-</sup> and mBMMC<sup>T+</sup> (figure 6.1). However, it is also possible that the semi-quantitative RT-PCR method used was insufficiently sensitive to detect small changes in transcription that may have occurred.

Reduced adhesion of mBMMC<sup>T+</sup> to fibronectin demonstrated in the previous chapter may have been due to TGF- $\beta_1$ -mediated down-regulation of  $\alpha 5$  expression. However, pretreatment with 5H10-27 only partially blocked adhesion of mBMMC<sup>T-</sup> to fibronectin, with median reductions in adhesion ranging from 19 - 52% in separate experiments using cells from different cultures (figure 6.6; appendix 2, table A2.9). This may have been due to expression of other fibronectin binding integrins, the balance between which may vary between cultures. In support of this, partial inhibition of IL-3-dependent mBMMC adhesion to fibronectin was attributed to co-expression of integrin  $\alpha 4$ , which was down-regulated in older cultures (Fehlner-Gardiner *et al*, 1996b). Further work would aim to investigate the role of  $\alpha 4$  in

mBMMC<sup>T-</sup> adhesion to fibronectin, however, these cells are phenotypically undefined and their role in this study was primarily as a comparator for mBMMC<sup>T+</sup>, as MMC homologues. Therefore exhaustive investigation of their adhesion mechanisms was not the main focus of this work.

Transcriptional down-regulation of expression of the integrin subunit  $\beta 3$ , which dimerises with  $\alpha v$  to form a vitronectin-binding integrin, may represent the mechanism of TGF- $\beta_1$ -mediated down-regulation of mBMMC adhesion to vitronectin. However, we showed only low expression of  $\alpha v$  on mBMMC<sup>T-</sup> by flow cytometry (figure 6.3), and use of a monoclonal anti- $\alpha v$  antibody did not significantly block mBMMC<sup>T-</sup> adhesion to vitronectin (figure 6.7). Therefore, although  $\alpha v \beta 3$  is the most likely candidate, the receptor mediating adhesion of mBMMC<sup>T-</sup> to vitronectin is unknown. These results are in contrast to those from similar investigations using IL-3-dependent mBMMC (Bianchine *et al*, 1992) and human skin mast cells (Columbo & Bochner, 2001), where adhesion to vitronectin was inhibited by anti- $\alpha v \beta 3$  polyclonal antibody and  $\alpha v$  expression was demonstrated by flow cytometry. The reason for this may be technical problems with the antibody, as no positive control was available, or it may be that  $\alpha v$  integrin does not play a role in adhesion of mBMMC<sup>T-</sup> to vitronectin.

As mentioned previously, the *in vivo* relevance of mBMMC<sup>T-</sup> is unknown, but they are likely to represent a more immature mast cell than mBMMC<sup>T+</sup>. If down-regulation of expression of integrins mediating adhesion to ECM proteins fibronectin and vitronectin occurs during differentiation from immature into mature MMC *in*



*vivo*, it could promote disengagement from the ECM of the lamina propria, possibly allowing intraepithelial migration. Thus, the combined TGF- $\beta_1$ -mediated down-regulation of  $\alpha 5$  and  $\beta 3$ , and the selective upregulation of the laminin-binding integrin  $\alpha 7$  may synergistically regulate intraepithelial migration of mast cells and maintenance of MMC intraepithelially.

## CHAPTER 7

### GENERAL DISCUSSION

Mast cells are an integral part of the immune response to gastrointestinal nematodes, and the aim of this work was to investigate the mechanisms regulating migration of MMC from the lamina propria into the jejunal epithelium following nematode infection.

The problem was addressed by focussing on three main hypotheses. The first hypothesis was that intestinal nematode infection would lead to upregulation of expression of possible mast cell chemoattractants by intestinal epithelium. The second hypothesis, therefore, was that MC would express receptors for chemokines expressed by intestinal epithelium, and that this expression is regulated during mast cell differentiation. The third hypothesis was that as TGF- $\beta_1$  regulates expression of the epithelial cell-binding integrin  $\alpha E$  (Miller *et al*, 1999), it could also regulate expression of ECM-binding integrins, which could play a role in intraepithelial migration of MMC.

The first hypothesis was addressed in chapter 3, which showed that mRNA for several potential mast cell chemoattractants was constitutively expressed by jejunal epithelium, including RANTES, MIP-1 $\alpha$ , fractalkine, SCF and TGF- $\beta_1$ ; TECK, which is chemotactic for IEL, was also expressed constitutively. Only MCP-1 was upregulated on *N. brasiliensis* infection, and high expression of this chemokine by

mBMMC<sup>T+</sup> was consistent with the hypothesis that upregulation of MCP-1 expression in intestinal epithelium was derived from influx of MMC.

The results of chapter 4 showed that mBMMC<sup>T+</sup> expressed mRNA for receptors corresponding to the chemokines expressed by intestinal epithelium; the receptors expressed included MIP-1 $\alpha$  and RANTES receptors CCR1 and CCR5, the MCP-1 – 5 receptor CCR2, and the fractalkine receptor CX3CR1. If mRNA is similarly expressed by MMC *in vivo*, and is translated to functional receptor protein, this would enable continuous migration of mast cells towards epithelium, implying that mast cell homing must be limited by other factors, as discussed in chapter 3.

However, before reaching this conclusion, further work would be required to show expression of chemoattractant and receptor proteins by jejunal epithelium and MMC. Commercially available ELISA kits could be used to investigate expression of chemokine protein (Li *et al*, 1998; Reale *et al*, 1998). Dual immunofluorescent staining for mMCP-1 and the receptor proteins would show receptor expression by MMC, though studies would be limited by the availability of anti-murine chemokine receptor antibodies and by fixation problems, as expression of mMCP-1 is difficult to show in frozen sections. Techniques such as tyramide amplification could be used to increase sensitivity of detection of sparsely expressed receptor proteins (Toda *et al*, 1999). *In situ*-hybridisation would be an alternative method for demonstrating the cells expressing receptor mRNA, although this would not prove protein expression.

Expression of functional receptor proteins by mBMMC<sup>T+</sup> could be shown by flow cytometry and chemotaxis assays, and investigation of migration of mBMMC<sup>T+</sup> into cultured intestinal epithelial monolayers could also be included. The majority of available chemokine receptor antibodies are not functionally-blocking, but the effect of specific small molecule antagonists such as met-RANTES, or of desensitising concentrations of chemokines (Phillips & Ager, 2002) on intraepithelial migration of mBMMC<sup>T+</sup> could be investigated.

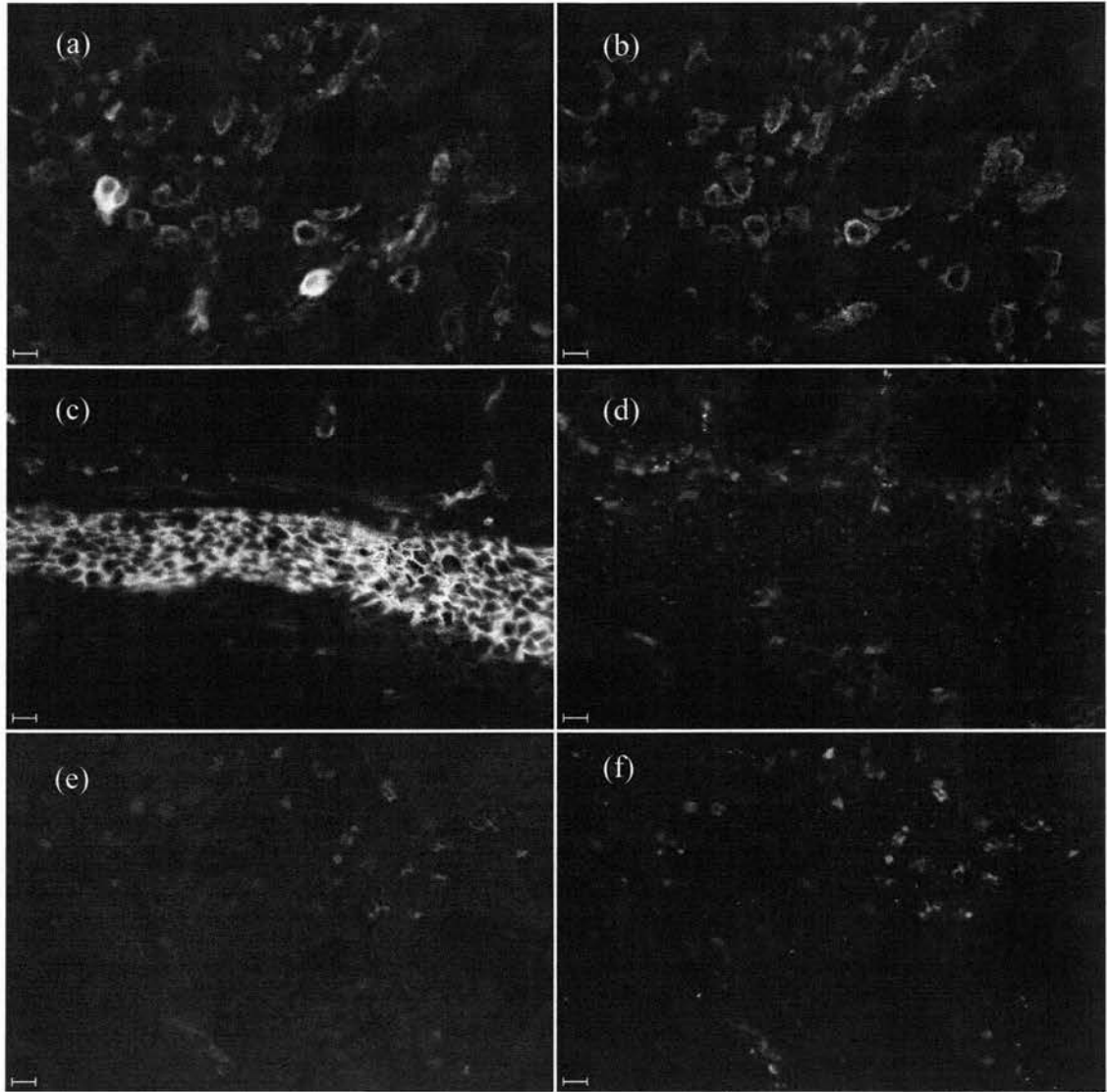
Our third hypothesis was that TGF- $\beta_1$  could regulate adhesion of mast cells to ECM proteins. TGF- $\beta_1$  did upregulate adhesion of mBMMC<sup>T+</sup> to the BM protein, laminin, via expression of integrin  $\alpha 7$ . This was unexpected as adhesion of IL3-dependent murine mast cells (Fehlner-Gardiner *et al*, 1996a; Vliagoftis & Metcalfe, 1997) and other leucocytes (Belkin & Stepp, 2000) to laminin is regulated by  $\alpha 6$  integrin.

The major role of  $\alpha 7$ , as discussed in chapter 6, is thought to be adhesion of skeletal myoblasts and mature skeletal myocytes to laminin, and the spectrum of adhesion is different to that of  $\alpha 6$ :  $\alpha 7$  isoforms adhere to laminins -1, -2/4 and -10/11 (von der Mark *et al*, 2002), but not to laminin-5. Although expression of  $\alpha 7$  by epithelial cells has been reported and is thought to participate in interactions with the BM (Basora *et al*, 1997), some authors state that laminin-5 is the major laminin isoform of BM *in vivo* (Falk *et al*, 1999; Erickson & Couchman, 2000), and that interactions of integrins  $\alpha 6\beta 1$  and  $\alpha 6\beta 4$  with laminin-5 regulate epithelial cell attachment (Belkin & Stepp, 2000).

The relevance of  $\alpha 7$  expression by mBMMC<sup>T+</sup> to intraepithelial maintenance of MMC is therefore debatable and, if it is expressed by MMC *in vivo*,  $\alpha 7$  may regulate aspects of mast cell function other than migration into jejunal epithelium. However, recent studies performed in our laboratory have co-localised expression of integrin  $\alpha 7$  and  $\alpha E$  in intraepithelially located cells of murine jejunum collected on day 9 of infection with *N. brasiliensis*, which are likely to be mast cells (figure 7.1). Fixation problems currently limit co-localisation with mMCP-1, but further studies using anti-IgE antibody would confirm the presumed identity of these cells, and strongly suggest some function for  $\alpha 7$  in intraepithelial MMC.

Use of  $\alpha 7$  knock-out mice (Mayer *et al*, 1997) would be central to further investigations of the role of  $\alpha 7\beta 1$  in intraepithelial migration or maintenance of MMC. Comparison of intraepithelial MMC numbers in these mice, to those in wild-type mice could determine the importance of  $\alpha 7$  in these processes.

Expression of  $\alpha 7$  by mBMMC<sup>T+</sup> is regulated by TGF- $\beta_1$ , and expression by MMC *in vivo* may also be regulated by this cytokine. *In vivo*, secreted TGF- $\beta_1$  must be activated to form a functional molecule, and epithelially-expressed integrin  $\alpha v\beta 6$  has been implicated in this process (Munger *et al*, 1999). Recent studies in our laboratory have shown co-expression of TGF- $\beta_1$  and integrin  $\alpha v\beta 6$  in murine jejunal epithelium, and that  $\beta 6$  knock-out mice have significantly reduced numbers of intraepithelial MMC following *N. brasiliensis* infection (Knight *et al*, 2002). This result is consistent with a role for TGF- $\beta_1$  in the intraepithelial MMC response to



**Figure 7.1. Integrin  $\alpha 7$  and  $\alpha E$  dual labelling of frozen sections of murine jejunum infected 9 days previously with *N. brasiliensis* .**

Confocal images of (a), (b), (e) and (f) jejunal epithelium and crypts; and (c), (d) jejunal smooth muscle.

$\alpha 7$  integrin was labelled with mouse anti-mouse- $\alpha 7$  IgG1 (clone 3C12)((a) and (c)) or isotype control (e).

$\alpha E$  integrin was labelled with rat anti-mouse  $\alpha E$  IgG2a (Becton Dickenson)((b) and (d)) or isotype control (f).

(a) and (b) show co-localisation of expression of integrins  $\alpha 7$  and  $\alpha E$  in jejunal epithelium, whereas, as expected, smooth muscle expresses  $\alpha 7$  but not  $\alpha E$ . Sections (e) and (f), labelled with isotype controls, are negative.

nematode parasites, and the mechanisms could include regulation of intraepithelial migration and retention of MMC via expression of  $\alpha 7\beta 1$ .

Use of mBMMC<sup>T+</sup> as MMC homologues has formed a large part of this study, therefore the suitability of mBMMC<sup>T+</sup> for study of the biology of MMC is an important consideration. These cells are, as described in 1.6.5 and 2.4.1, cultured in the presence of four cytokines IL-3, IL-9, SCF and TGF- $\beta_1$ , which result in differentiation into cells resembling MMC in terms of both morphology and expression of mMCP-1. However, *in vivo*, mast cell differentiation is likely to occur in the presence of a number of cytokines and other molecules, some of which including IL-4 (Hamaguchi *et al*, 1987), IL-6 (Yuan *et al*, 1998) and IL-10 (Thompson-Snipes *et al*, 1991; Yuan *et al*, 1998), regulate mast cell differentiation. mBMMC<sup>T+</sup> are known to resemble MMC in some respects, but the extent of this homology with respect to adhesion molecules remains to be confirmed.

As discussed in chapter 4, the culture system used is not suitable for examination of MC precursors, which would confirm the trends in chemokine receptor expression suggested in chapter 4, and enable investigation of the chemokines regulating recruitment of mast cell precursors into the jejunal mucosa. Further work would include development of an assay to determine if mast cell precursors from a population of freshly isolated murine bone marrow cells migrated towards the relevant chemokines; this would confirm expression of functional receptor proteins. Enrichment for mast cell precursors in migrated cells could be shown by comparison of % MMC in migrated versus non-migrated populations.

If expression of the molecules described does regulate intraepithelial migration of MMC *in vivo*, how then would these mechanisms compare with those of other cells? The most obvious and relevant comparison would be that of IEL, which also reside in the intestinal epithelium. IEL and mBMMC<sup>T+</sup> share expression of some chemokine receptors including CCR2 (Agace *et al*, 2000), CCR5 (Agace *et al*, 2000; Shires *et al*, 2001), CX3CR1 (Muehlhoefer *et al*, 2000) and CXCR4 (Agace *et al*, 2000), suggesting common recruitment mechanisms. However, as discussed in chapter 4, mBMMC<sup>T+</sup> do not express CCR9, which is thought to be critical to small intestinal recruitment of T lymphocytes (Kunkel *et al*, 2000), suggesting possibly that mast cells and IEL share expression of receptors for chemokines expressed in inflammation, but not those reputedly involved in trafficking.

Both IEL and mBMMC<sup>T+</sup> express the integrin  $\alpha E\beta 7$  in a TGF- $\beta_1$ -dependent fashion, which may maintain both cell-types intraepithelially, but investigations of adhesion of IEL to ECM proteins suggests these two cells-types may differ in this respect. In one study (Roberts *et al*, 1999), IEL adhered strongly to type IV collagen compared to peripheral blood CD8<sup>+</sup> T cells; antibodies to integrins  $\alpha 1$  and  $\beta 1$  blocked this adhesion. Adhesion of IEL to type IV collagen was hypothesised to support maintenance of these cells intraepithelially and, in support of this, there was a 50% reduction of IEL in VLA1 ( $\alpha 1\beta 1$ ) null mice (Meharra *et al*, 2000). mBMMC<sup>T+</sup> adhered to type IV collagen in some experiments (appendix 2, table A2.3), but this was inconsistent, and these cells adhered most avidly to laminin. In contrast, IEL adhered poorly to laminin (Roberts *et al*, 1999), and showed low expression of



integrin  $\alpha 6 \beta 1$ , the major laminin receptor for IL3-dependent mBMMC and T-lymphocytes. To our knowledge, expression of the integrin  $\alpha 7$  has not been shown in IEL, though a recent study of gene expression in IEL using SAGE analysis demonstrated strong expression of LBP, and integrins  $\alpha E$  and  $\beta 7$  (Shires *et al*, 2001). Therefore, if a role for  $\alpha 7$  integrin is shown in intraepithelial location of MMC, it is possible that IEL and MMC interact with the epithelial BM by different mechanisms. Since MMC but not IEL are recruited following infection of mice with *N. brasiliensis* (Knight *et al*, 2002), some differences in the recruitment mechanisms of these two cell types might be expected.

This study suggests that, as in other leucocytes and mast cell phenotypes, interplay between integrins and chemokines is likely to regulate migration of MMC and their precursors. It has also shown that mBMMC<sup>T+</sup> adhere to laminin-1 through expression of  $\alpha 7$  integrin, which has not previously been demonstrated in a haemopoietic cell. The physiological significance of this finding is yet to be determined, but this and other aspects of this work have opened up many avenues of further investigation of the migratory mechanisms and biological functions of MMC.

## APPENDIX 1

Preparation of solutions, buffers and substrates.

### 10 x PBS

85.0 g NaCl  
27.0 g  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$   
3.9 g  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$

Dissolve in 1 litre of deionised water and dilute x 10 before use.

### CARNOY'S FIXATIVE

60 ml 100% ethanol  
30 ml chloroform  
10 ml glacial acetic acid

### 20 X SALINE SODIUM CITRATE (SSC) (3 M NaCl / 0.3 M sodium citrate)

175.3 g NaCl  
88.2 g sodium citrate

Dissolve in 1 litre of deionised water and pH to 7.5 with HCl

Dilute as necessary to make SSC and SSC / SDS solutions used for washes during Southern blotting.

### MALEIC ACID BUFFER (0.15 M NaCl / 0.1 M Maleic acid)

8.8 g NaCl  
11.6 g Maleic acid

Dissolve in 1 litre of deionised water and adjust pH to 7.5 with solid NaCl then concentrated NaCl.

### CARBONATE BUFFER FOR ELISAS

- A. 0.1 M  $\text{NaHCO}_3$  - Dissolve 0.84 g  $\text{NaHCO}_3$  in 100 ml deionised water
- B. 0.1 M  $\text{NaCO}_3$  - Dissolve 1.06 g  $\text{NaCO}_3$  in 100 ml deionised water

Add solution B to solution A until the pH reaches 9.6.

## SCOTT'S TAP WATER

1 litre tap water  
3.5 g NaHCO<sub>3</sub>  
20.0 g MgSO<sub>4</sub>·7H<sub>2</sub>O

Adjust pH to 8.0

## SUBSTRATE BUFFER FOR $\beta$ -HEXOSEAMINIDASE ASSAY (7.5 mM p-nitrophenol-N- $\beta$ -glucoseaminide)

- A. 0.1 M citrate buffer, pH 5.0 - Dissolve 5.25 g citric acid monohydrate in 250 ml of deionised water, pH to 5.5 with NaOH.  
B. 0.5% triton -X 100 in deionised water

Dissolve 0.052g of 7.5 mM p-nitrophenol-N- $\beta$ -glucoseaminide in 20 ml of buffer A, heating gently and stirring. Add 20 ml of solution B, aliquot and store at -20°C.

## SOLUBILISATION OF PROTEINS USED FOR COATING OF ELISA PLATES IN ADHESION ASSAYS

Proteins were solubilised and stored as recommended by Sigma (table A1.1).

Protein	Solubilisation method
Rat tail type I collagen	Solubilised at 1 mg/ml in sterile distilled H <sub>2</sub> O with 0.1% glacial acetic acid, agitating gently for 1 h, and stored undiluted at 4°C before use.
Human placenta type IV collagen	Solubilised at 1 mg/ml in sterile distilled H <sub>2</sub> O, agitating gently, and stored undiluted at 4°C before use.
Bovine fibronectin	Solubilised at 1 mg/ml in sterile distilled H <sub>2</sub> O by leaving to stand at 37°C for 30 min and stored undiluted at 4°C.
Murine Engelbreth-Holm-Swarm sarcoma laminin-1	Laminin-1 was provided in a 1 mg/ml solution in Tris-buffered saline. Before use, it was aliquoted and stored at -20°C.
Bovine plasma vitronectin	Solubilised at 50 $\mu$ g/ml in sterile distilled H <sub>2</sub> O and stored undiluted at 4°C before use.

**Table A1.1. Solubilisation of ECM proteins, all obtained from Sigma.**

## SOLUBILISATION OF STIMULATING AGENTS USED FOR ADHESION ASSAYS

### PHORBOL MYRISTATE ACETATE (PMA)

PMA was dissolved at 1 mg/ml in DMSO and stored at -20°C in the dark. For adhesion assays, PMA in DMSO was diluted 1:1000 in PBS and 5 µl added to each well to make a final concentration of 50 ng/ml.

### CALCIUM IONOPHORE A23187

Calcium ionophore was dissolved at 1 mg/ml in DMSO and stored at room temperature. For adhesion assays, calcium ionophore in DMSO was diluted 1:100 in PBS and 5 µl added to each well to make a final concentration of 1 µM.

## APPENDIX 2

Results of repeat experiments in chapters 5 and 6.

Expt	BSA		Coll I		Coll IV		Fibronectin		Vitronectin		Laminin-1	
	1 h	2 h	1 h	2 h	1 h	2 h	1 h	2 h	1 h	2 h	1 h	2 h
1	0.3 (0.2–0.4)	0.0 (0.0–0.0)	0.4 (0.3–0.6)	0.0 (0.0–0.0)	1.0 (0.3–1.1)	0.0 (0.0–0.0)	5.3 (4.3–6.0)	0.0 (0.0–0.0)	0.5 (0.3–0.9)	0.0 (0.0–0.0)	42.2 (38.3–58.1)	26.6 (25.0–54.5)
2	0.0 (0.0–0.0)	ND	0.0 (0.0–0.0)	ND	0.0 (0.0–0.0)	ND	1.3 (0.1–2.3)	ND	0.0 (0.0–0.0)	ND	58.6 (55.4–67.4)	ND
3	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	42.6 (31.8–43.8)	14.6 (14.5–17.6)

**Table A2.1. Experiment 5.2 - investigation of adhesion of unstimulated mBMMC<sup>T+</sup> to ECM proteins.**

mBMMC<sup>T+</sup> were incubated for 1 or 2 h in wells coated with 20 µg/ml of ECM proteins.

Results are expressed as median (range) n=3 wells of % mBMMC adherent. Values < 0 were assigned as 0.

ND = Not done in this experiment.





Expt.	n	Control	Ag	IgE	Ag/IgE	A23187	PMA
1	3	0.0 (0.0-0.0)	ND	ND	22.5 (17.6-25.3)	ND	33.1 (27.2-45.0)
2	4	6.1 (2.5-8.2)	7.3 (6.7-14.7)	16.8 (14.9-19.4)	32.7 (21.4-48.6)	17.3 (15.0-36.3)	42.4 (27.9-63.6)
3	4	19.4 (13.3-32.5)	25.4 (19.4-46.0)	17.4 (13.0-40.1)	12.5 (8.8-18.3)	26.8 (18.3-51.4)	47.7 (21.2-90.2)
4	4	11.1 (5.1-21.1)	10.1 (8.0-12.4)	8.9 (0.0-27.5)	28.8 (18.5-36.7)	21.4 (18.8-21.7)	49.4 (35.0-60.3)
5	3	19.9 (19.1-27.8)	ND	ND	23.8 (21.0-33.8)	31.6 (20.8-33.8)	38.8 (36.5-42.1)

**Table A2.4. Experiment 5.9 - regulation of the adhesion of mBMMC<sup>T+</sup> to laminin-1.**

Data are from 5 experiments investigating mechanisms stimulating adhesion of mBMMC<sup>T+</sup> to laminin-1. Results are expressed as median (range) of % mBMMC adherent. ND = not done in this experiment. Values <0 were assigned as 0.



Expt.	n	Control	Ag	IgE	Ag/IgE	A23187	PMA
1	3	1.6 (1.4 - 4.8)	ND	ND	67.0 (52.5 - 76.4)	ND	77.3 (72.4 - 78.2)
2	4	31.6 (14.1 - 41.4)	19.5 (9.2 - 36.6)	7.0 (4.3 - 27.0)	44.7 (25.7 - 61.3)	45.8 (29.6-57.8)	93.8 (67.4 - 100.0)
3	4	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.2)	33.8 (31.1 - 37.2)	0.0 (0.0 - 37.2)	74.4 (69.3 - 75.7)

**Table A2.5. Experiment 5.9 - regulation of the adhesion of mBMMC<sup>T-</sup> to fibronectin.**

Results of 3 experiments investigating mechanisms stimulating adhesion of mBMMC<sup>T-</sup> to fibronectin. Data are expressed as median (range) of % mBMMC adherent. ND = not done in this experiment. Values <0 were assigned as 0.

Expt.	n	Control	Ag	IgE	Ag/IgE	A23187	PMA
1	3	0.0 (0.0 - 0.0)	ND	ND	38.9 (27.5 - 43.2)	ND	58.0 (50.0 - 66.8)
2	4	0.0 (0.0 - 0.0)	ND	ND	10.3 (4.9 - 14.0)	3.3 (1.5 - 4.4)	43.7 (38.8 - 48.9)

**Table A2.6. Experiment 5.9 - regulation of the adhesion of mBMMC<sup>T-</sup> to vitronectin.**

Results of 2 experiments investigating mechanisms stimulating adhesion of mBMMC<sup>T-</sup> to vitronectin. Data are expressed as median (range) of % mBMMC adherent. ND = not done in this experiment. Values <0 were assigned as 0.

Expt.	n	6A11	IgG1	No antibody	% reduction
1	3	0.0 (0.0 – 0.0)	52.7 (48.4 – 60.5)	63.8 (57.2 – 79.6)	100
2	4	1.5 (1.2 – 2.5)*	65.4 (59.3 – 69.0)	59.4 (54.7 – 72.3)	97.7
3	4	0.2 (0.0 – 0.4)*	80.9 (80.4 – 85.5)	73.7 (69.3 – 77.7)	99.8

**Table A2.7. Antibody blocking experiment 6.5 - 6A11.**

Results of 3 experiments investigating the effect of the anti- $\alpha 7$  monoclonal 6A11 or IgG1 control (10  $\mu\text{g/ml}$ ) on adhesion of mBMMC<sup>T+</sup> to laminin-1.

Preincubation with 6A11 results in a significant ( $p<0.05$ )(\*) reduction in adhesion of mBMMC<sup>T+</sup> to laminin, compared to cells incubated with IgG2a control, or no antibody. Data are expressed as median (range) of % mBMMC adherent. Values <0 were assigned as 0.

	Expt.	n	GoH3	IgG2a	No antibody	% reduction
MC/9	1	3	0.7 (0.7 – 0.8)	36.5 (34.3 – 47.0)	43.8 (40.8 – 48.5)	98.1
	2	4	2.1 (2.0 – 2.8)*	30.1 (22.6 – 47.6)	39.4 (35.9 – 40.5)	94.4
	3	4	1.7 (0.7 – 2.7)*	71.7 (68.6 – 74.9)	76.5 (74.3 – 80.0)	97.6
mBMMC <sup>T+</sup>	1	3	68.5 (59.5 – 73.1)	70.8 (61.3 – 70.8)	62.6 (59.4 – 67.5)	3.2
	2	4	85.8 (76.9 – 100.0)	84.5 (61.3 – 88.7)	77.3 (73.3 – 79.0)	-1.5
	3	4	60.1 (53.7 – 73.2)	59.2 (51.2 – 75.3)	45.7 (41.7 – 64.2)	-1.5

**Table A2.8. Antibody blocking experiment 6.5 - GoH3.**

Results of 3 experiments investigating the effect of the anti- $\alpha 6$  monoclonal GoH3 or IgG2a control (1  $\mu\text{g/ml}$ ) on adhesion of MC/9 cells or mBMMC<sup>T+</sup> to laminin-1.

Preincubation of MC/9 cells with GoH3 resulted in a significant ( $p < 0.05$ )(\*) reduction in adhesion to laminin-1 compared to cells incubated with IgG2a control, or no antibody. In contrast, incubation of mBMMC<sup>T+</sup> with GoH3 under the same conditions did not inhibit adhesion to laminin-1. Results are expressed as median (range) of % mBMMC adherent. Values >100 were assigned as 100.

Expt	Ab conc. (µg/ml)	5H10-27	IgG2a	No antibody	% reduction
1	1	23.7 (15.9 – 26.3)*	49.4 (46.4 – 56.1)	56.5 (32.5 – 63.4)	52.0
1	5	22.4 (17.3 – 27.7)*	43.7 (39.5 – 48.0)	56.5 (32.5 – 63.4)	48.7
1	10	16.8 (14.4 – 51.5)	48.2 (44.2 – 55.0)	56.5 (32.5 – 63.4)	65.1
2	10	46.7 (39.9 – 48.3)*	57.7 (54.6 – 62.2)	ND	19.0
3	10	43.5 (37.2 – 46.9)*	59.4 (57.2 – 60.6)	50.8 (44.6 – 56.2)	26.8
4	10	40.7 (16.7 – 58.4)	63.6 (44.3 – 69.1)	63.1 (50.6 – 72.2)	36.0

**Table A2.9. Antibody blocking experiment 6.5 - 5H10-27.**

Results of 4 experiments investigating the effect of the anti-α5 monoclonal 5H10-27 or IgG2a control on adhesion of mBMMC<sup>T</sup> to fibronectin.

\* indicates a significant difference (p<0.05) between adhesion of mBMMC<sup>T</sup> incubated with 5H10-27 and IgG2a. Data are expressed as median (range) of % mBMMC adherent, n=4 wells. ND = not done in this experiment.

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## PUBLICATIONS ARISING FROM THIS THESIS

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## Chemokine and cytokine expression in murine intestinal epithelium following *Nippostrongylus brasiliensis* infection

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### SUMMARY

*Infection of mice with the nematode parasite Nippostrongylus brasiliensis results in a well characterized intestinal mastocytosis with intraepithelial migration of mucosal mast cells (MMC). The molecules mediating this response are unknown. We examined expression of several putative mast cell chemoattractants in intestinal epithelium following N. brasiliensis infection. Expression of the chemokines monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), RANTES (regulated on activation normal T-cell expressed and secreted), fractalkine, and thymocyte expressed chemokine (TECK); and the cytokines stem cell factor (SCF) and transforming growth factor  $\beta_1$  (TGF $\beta_1$ ), was constitutive and no alteration was detected following infection. MCP-1 expression was also constitutive but at much lower levels and increased expression was detected on days 7 and 14 postinfection. Expression of MCP-1 in whole jejunum was at much higher levels than in epithelium. Constitutive expression of MCP-1, MIP-1 $\alpha$  and TGF $\beta_1$  was also detected in cultured bone marrow-derived homologues of MMC. In an intestinal epithelial cell line (CMT-93), there was constitutive expression of SCF, TGF $\alpha_1$ , fractalkine and MCP-1. The results show that, in vivo, epithelium is a potentially important source of mast cell chemoattractants.*

**Keywords** *Nippostrongylus brasiliensis*, mast cells, epithelium, chemokines

### INTRODUCTION

As the first point of host contact, the epithelium is well placed for an important role in the immune response to gut-dwelling pathogens including nematode parasites. Expression of chemoattractant molecules by epithelial or intraepithelial cells may initiate the immune response by attraction of the necessary spectrum of immune cells for parasite expulsion, and recent attention has focused on chemokines as co-ordinators of the immune response by specific recruitment of inflammatory cells. These molecules are a structurally related group of 8–10 kDa inducible cytokines that are chemotactic for a variety of immune cells, including dendritic cells (1), T-cells, monocytes, neutrophils (2,3) and mast cells (4–7), and are up-regulated in inflammatory diseases.

There is considerable evidence that modulation of chemokine expression plays an important role in the epithelial response to intestinal infections. Interaction of invasive bacteria stimulates up-regulation of monocyte chemoattractant protein-1 (MCP-1) and interleukin (IL)-8 in cultured colon epithelial cells (8) and rotavirus infection of cultured intestinal epithelium stimulates up-regulation of macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), MCP-1 and RANTES (regulated on activation normal T-cell expressed and secreted) (9). Also, stimulation of epithelium by pro-inflammatory cytokines such as IL-1 and tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) can up-regulate chemokine expression (10).

Less is known about the intestinal epithelial response to gut-dwelling parasites, but studies using both cell culture (11) and epithelial isolation methods (12) have shown increases in IL-1 $\beta$ , IL-8 and epithelial-neutrophil activating peptide-78 (ENA-78) in response to infection with the nematode parasite, *Trichinella spiralis*. The epithelial cytokine and chemokine response to *Nippostrongylus brasiliensis* infection has not been studied but the cellular response to this nematode is well characterized; infection of mice with *N. brasiliensis* L3 larvae results in an intestinal mastocytosis with intraepithelial migration (13). Since a number of

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chemokines are chemotactic for certain mast cell phenotypes (4–7,14), it is possible that epithelially derived chemokines could mediate this migration. In addition to this, the cytokines stem cell factor (SCF) and transforming growth factor  $\beta_1$  (TGF $\beta_1$ ), which may also be expressed by epithelium (15,16), are both potential mast cell chemoattractants (17,18). We therefore examined expression of the putative mast cell chemoattractants MIP-1 $\alpha$ , MCP-1, RANTES, fractalkine, SCF and TGF $\beta_1$ ; along with the T-cell chemoattractant thymocyte expressed chemokine (TECK), in intestinal epithelium following *N. brasiliensis* infection. The expression of these cytokines and chemokines was compared with that of the mucosal mast cell-specific granule chymase, mouse mast cell protease-1 (mMCP-1). This chymase is uniquely expressed by intraepithelial mucosal mast cells (MMCs) (19) and is released systemically and into the gut lumen. We have also introduced a newly adapted method of isolating intestinal epithelium, which allows analysis of the epithelial immune response *in vivo*.

## MATERIALS AND METHODS

### Collection of whole jejunum and jejunal epithelial samples

The mouse-adapted strain of the nematode parasite *N. brasiliensis* was maintained as described previously (20) and 500 L3 stage larvae were used to infect anaesthetized 12-week-old male BALB/c mice (Bantam and Kingman Universal, Hull, UK) by subcutaneous injection. Infection of mice in this way has previously been shown to produce a prolonged infection resulting in measurable faecal egg counts from day 6, with worms being detectable in the intestines of mice 5 days after infection (19). Mice were killed in groups of three and samples collected from uninfected mice, and from mice 1, 2, 4, 7 or 14 days after infection. A solution of 24 mM ethylenediaminetetraacetic acid (EDTA) in Calcium-Magnesium-free Hank's Balanced Salt Solution (CMF HBSS) (Gibco, Paisley, UK), pH 7.4 was prepared immediately before experiments.

Anaesthetized mice were bled and serum retained for analysis of mMCP-1 levels by ELISA as previously described (19) before being killed by careful cervical dislocation. Epithelium was then stripped from a section of proximal jejunum using a modification of the method previously described by Bjerknes *et al.* (21). The abdomen was opened and a 10-cm length of proximal jejunum was flushed free of intestinal contents using warm CMF HBSS. The thorax was then opened and after sectioning the caudal vena cava at the diaphragm, mice were perfused at a rate of approximately 10 ml/min via the left ventricle with 90 ml of 24 mM EDTA in CMF HBSS at 37°C using a syringe driver (Harvard Apparatus Ltd, Edenbridge, UK). Following perfusion, the

gut segment was removed and a 2-cm piece placed into RNA later (Ambion, Austin, Texas USA) for at least 24 h before homogenization, on ice, into TRI reagent (Sigma, Poole, UK). The remainder was divided into two segments, which were tied at one end onto a thin metal rod and then everted. The epithelium was then released from the underlying tissue by rapid manual rotation of the gut segment in ice cold CMF HBSS. The epithelial fragments were allowed to settle on ice before removing into cold TRI reagent. Some of the exfoliated epithelium was placed in 4% paraformaldehyde/PBS for examination by light microscopy and histological examination in order to check the purity of the cell population collected (Figure 1, Table 1). After 24 h in paraformaldehyde, samples were placed in 70% alcohol before initial embedding in agar, followed by routine processing into paraffin wax. Sections, 4  $\mu$ m thick, were stained using routine haematoxylin and eosin protocols.

### Bone marrow mast cell culture

Bone marrow mast cells were cultured in the presence of recombinant (r) IL3 or WEHI, rIL9, rSCF and rTGF $\beta_1$  as described previously (22).  $1 \times 10^7$  cells cultured for 14–21 days were placed into TRI reagent for RNA extraction. These cells were > 95% mast cells containing mMCP-1 and with a morphology resembling mucosal mast cells (23) as described.

### Culture of CMT-93 murine intestinal epithelial cells

The epithelial cell line CMT-93, derived from a mouse rectal carcinoma, was a gift from Dr P.J. Kilshaw (Babraham Institute, Cambridge, UK). Cells were seeded at  $1\text{--}3 \times 10^4$ /ml in Dulbecco's minimum essential medium (Gibco) with 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin (Sigma) and 10% foetal calf serum (Serotec, Kidlington, UK) and grown to confluence before harvesting into TRI reagent.

### Detection of transcripts by reverse transcription-polymerase chain reaction (RT-PCR)

Expression of MCP-1, MIP-1 $\alpha$ , RANTES, TECK, fractalkine, TGF $\beta_1$  and SCF in jejunal epithelial samples, cultured mast cells and cultured CMT-93 cells; and of MCP-1 in whole jejunum and mMCP-1 in epithelium only, was measured by RT-PCR. Total RNA was recovered from samples in TRI reagent by chloroform extraction and precipitation according to the manufacturer's instructions for TRI reagent. RNA quality and quantity was then determined by measurement of absorbance at 260/280 nm in a Beckman DU 650 spectrophotometer. To eliminate contaminating genomic DNA, 20  $\mu$ l aliquots of each RNA sample were DNAsed using a 'DNA-free' kit (Ambion) according to the



manufacturer's instructions; samples were then re-quantified by spectrophotometry as before.

1 µg of each RNA sample was reverse-transcribed in a 20-µl volume using a Promega Reverse Transcription (RT) kit (Promega, Southampton, UK). Incubation was for 1 h at 42°C followed by 5 min at 99°C. 1/20th volume of cDNA was used for PCR using gene-specific primers (Table 2) which were intron-spanning where possible. cDNA was amplified using 35 cycles of 94°C for 30 s, 50°C (fractalkine), 55°C (TECK, RANTES, SCF), 60°C (MCP-1) or 63°C (mMCP-1) for 30 s and 72°C for 1 min. For each sample, primers for GAPDH were included as a positive control and negative controls were also set up containing RNA only (no cDNA). PCR products were separated on a 1.4% agarose gel containing 0.5 µg/ml ethidium bromide and visualized and recorded under ultraviolet light using a Kodak Image Station 440cf imaging system. PCR product identities were confirmed by Southern hybridization using gene-specific oligonucleotide probes as described previously (24). Hybridized digoxigenin (DIG)-labelled hybridized probes were detected with an anti-DIG alkaline phosphatase antibody using chemiluminescence with CDP-star (Hoffmann-La Roche Co. Ltd. Basel, Switzerland), and images were visualized and recorded using the Kodak Image Station 440cf imaging system.

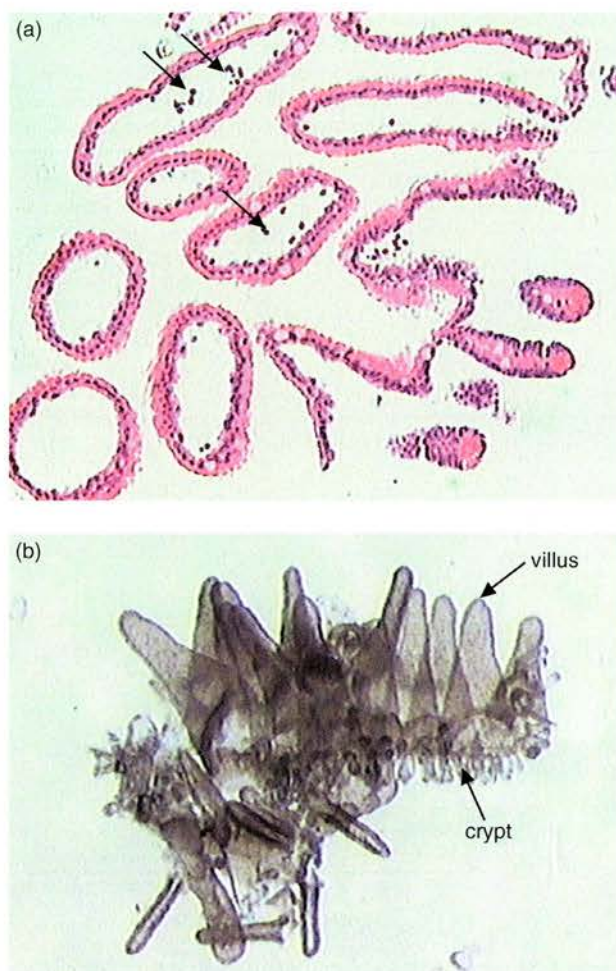
#### Semi-quantitative analysis of MCP-1 expression in jejunal epithelium

Since initial experiments suggested increased MCP-1 expression in jejunal epithelium on day 7 postinfection, this was further investigated using semiquantitative PCR. Low levels of MCP-1 expression in epithelial samples necessitated use of 10-fold more cDNA (1 : 2 volume of RT reactions) in each PCR reaction. The correct cycle number was determined so that PCR reactions were not reaching saturation, and samples were amplified for 34 cycles as previously described for MCP-1. To control for the amount of cDNA in each sample, 1 : 20 of volume of cDNA was amplified for 20 cycles using primers for GAPDH. As before, the number of cycles of amplification was chosen so that the reaction was not approaching saturation. PCR products were separated and visualized as before and the ratios of intensities of signals for MCP-1 and GAPDH were taken to represent relative MCP-1 expression.

## RESULTS

#### Examination of stripped epithelium

Examination of stripped epithelium under low power using a dissecting microscope showed that collected samples



**Figure 1** Jejunal epithelial samples from epithelial stripping experiments demonstrating that intact crypt-villous units have been isolated. (a) Haematoxylin and eosin-stained epithelial preparations showing the relative absence of non-epithelial elements ( $\times 100$ ). Arrows indicate contaminating non-epithelial cells. (b) Isolated sheet of epithelium ( $\times 25$ ).

consisted of predominantly epithelium either in small sheets, or as discrete crypt-villous units or separated crypts and villi (Figure 1). Examination of haematoxylin and eosin-stained histological sections confirmed this, levels of contaminating nonepithelial cells were below 5% (Table 1). Differential expression of MCP-1 in whole jejunal and epithelial samples (see below) further supported evidence that these represented two different cell populations.

#### mMCP-1 expression in epithelium

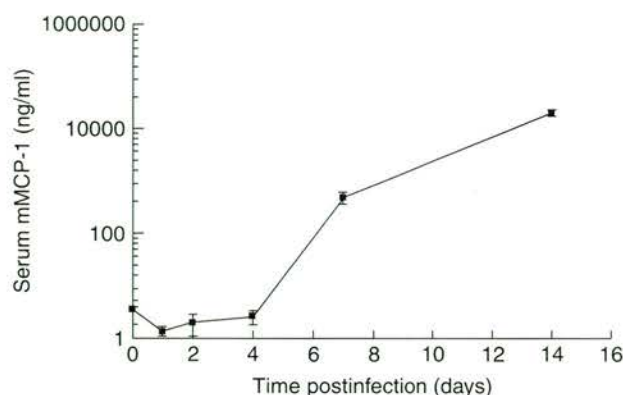
In order to confirm a mastocytosis (and therefore successful infection and migration of *N. brasiliensis* to the gut), serum



**Table 1** Contamination of epithelial preparations with other cell types. Five hundred epithelial cells were counted in each haematoxylin and eosin-stained preparation examined, and the number of associated nonepithelial cells estimated. Contaminating cells consisted mainly of lymphocytes with occasional neutrophils

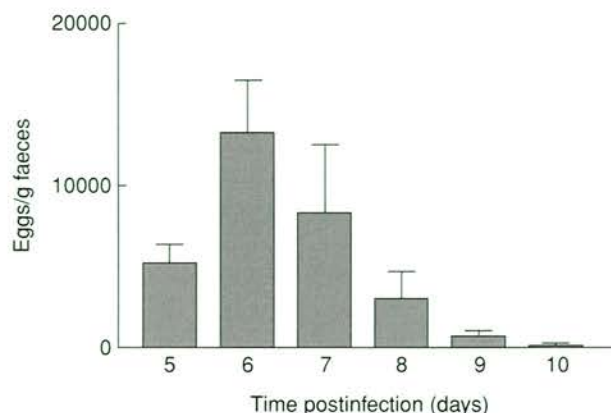
Time postinfection (days)	<i>n</i>	Mean percentage contaminating cells
0	3	4.4
1	3	2.3
2	2	3.0
4	3	2.5
7	3	3.1
14	2	1.8

*n*, Number of preparations examined.



**Figure 2** Serum mMCP-1 concentrations in male BALB/c mice following *N. brasiliensis* infection measured by ELISA. Values are the mean  $\pm$  SEM for three samples.

levels of mMCP-1 were measured. mMCP-1 was detected in the serum of all mice, but at very low levels up to day 4 (mean serum concentration = 2.6 ng/ml day 4 postinfection). Serum mMCP-1 concentrations (mean of three mice) were increased above controls in mice from day 7 (0.5  $\mu$ g/ml) and day 14 (20  $\mu$ g/ml) postinfection (Figure 2). As previously reported (19,24), the rise in mMCP-1 negatively correlates with faecal egg counts in this case from a repeat experiment using the same strains of mice and parasites (Figure 3). To specifically confirm movement of mast cells into the epithelium, expression of mMCP-1 was measured in epithelial samples by RT-PCR. Expression was detected in all samples collected on days 7 and 14 postinfection (Figure 4); an almost undetectable level of expression was seen in some samples on days 0, 1 and 2 postinfection. This is consistent with a previous study of whole jejunal mMCP-1 expression using Northern blotting (25).



**Figure 3** Faecal egg counts after infection of male 12-week-old BALB/c mice with 500 L<sub>3</sub> stage larvae of the mouse-adapted strain of *N. brasiliensis*. Results are the mean  $\pm$  SEM of egg counts from three mice.

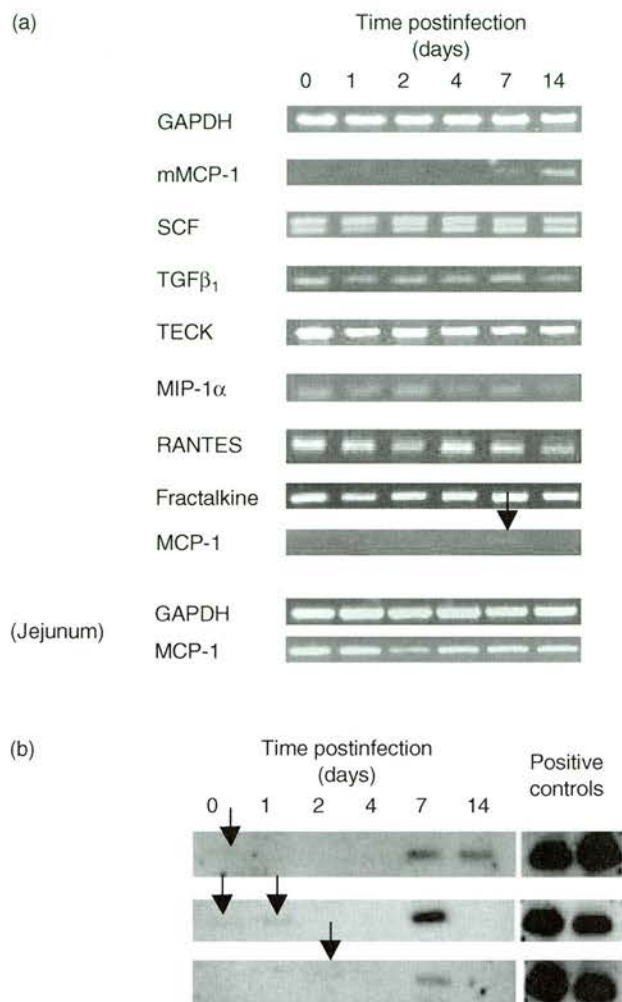
### Chemokine and cytokine expression in jejunal epithelium

After subcutaneous injection, *N. brasiliensis* migrates through the lungs arriving in the intestine between 48 h and 72 h after infection. Numbers of MMCs begin to increase on day 7 postinfection but, in order to examine both early and late responses, expression of chemokines and cytokines was examined (in triplicate) on days 1, 2, 4, 7 and 14 postinfection. Using RT-PCR, expression of the chemokines MIP-1 $\alpha$ , RANTES, TECK and fractalkine, and of the cytokines SCF and TGF $\beta$ <sub>1</sub>, was detected in both control (uninfected) epithelial samples and in epithelial samples collected at all time-points from infected mice (Figure 4). No consistent changes in expression were detected using this method. All PCR products were the expected size and hybridized, as expected, to complementary oligonucleotide probes (Table 2).

Expression of the chemokine MCP-1 was initially undetectable by RT-PCR in all except samples collected on day 7 postinfection and in one sample collected on day 14 postinfection (Figure 4). Southern blotting detected low levels of expression in two control samples, two samples from day 1 postinfection and in one day 2 sample, and confirmed the increased levels of expression in all day 7 samples and one day 14 sample. Use of 10-fold more cDNA for semi-quantitative PCR showed low levels of MCP-1 expression in all samples, with increased expression in two samples collected on day 7 and 2 samples from day 14 postinfection (Figure 5).

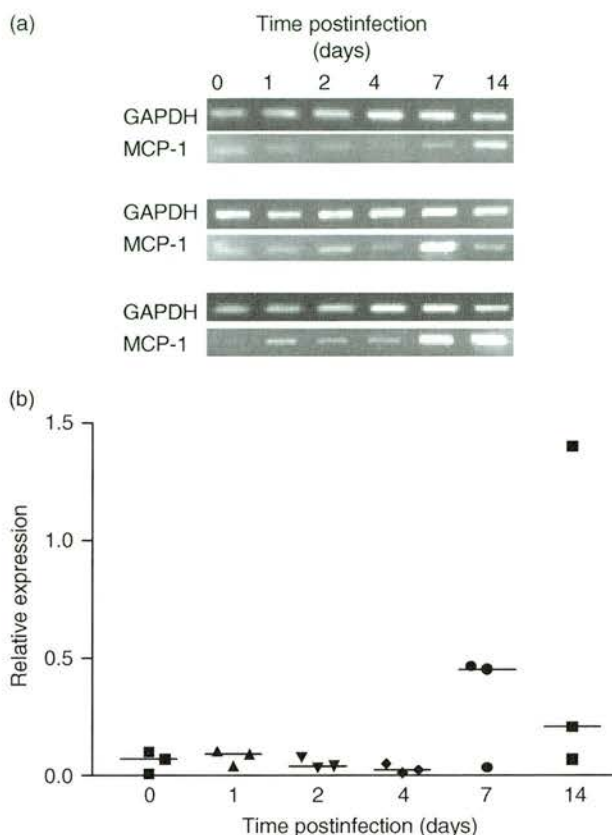
### MCP-1 expression in whole jejunum

As little MCP-1 was initially detected in epithelial samples, expression of MCP-1 was examined in whole jejunal samples collected alongside epithelial samples.



**Figure 4** Expression of cytokines and chemokines in mouse jejunal epithelial samples following *N. brasiliensis* infection. (a) RT-PCR expression of mMCP-1 on days 7 and 14 confirms movement of mast cells into the epithelium as a result of *N. brasiliensis* infection. All chemoattractants are constitutively expressed at levels detectable by RT-PCR with the exception of MCP-1, which is detected only on day 7 (shown by arrow) in this example. For comparison, MCP-1 expression in whole jejunal samples is shown, which is constitutive and appears to be at much higher levels than epithelial expression. Results are representative of data from three separate mice. Upper and lower bands of the SCF signal represent secreted and transmembrane forms respectively. (b) Southern blot of PCR products from amplification using primers specific for MCP-1. Arrows indicate faint bands. Positive controls are whole jejunal samples, showing much higher expression of MCP-1.

In contrast to that seen in epithelium, expression was detected by RT-PCR in control samples, and at all time points postinfection (Figure 4). No increase in expression was seen following *N. brasiliensis* infection. Expression of



**Figure 5** Semi-quantitative PCR analysis of MCP-1 expression in jejunal epithelium following *N. brasiliensis* infection. (a) Ethidium bromide-stained gels showing MCP-1 and respective GAPDH signals. (b) Scatter graph of semi-quantitative MCP-1 expression in jejunal epithelium. Values are the ratios of intensity of MCP-1 signal/intensity of GAPDH signal. Horizontal bars indicate median values.

MCP-1 in whole jejunal samples was at higher levels than in epithelium (Figure 4).

#### Chemokine expression in cultured mucosal mast cells and CMT-93 cells

In order to investigate possible sources of the chemokines and cytokines expressed from intestinal epithelial samples, we examined (in triplicate) expression in a cultured mouse rectal epithelial cell line (CMT 93) and in cultured MMC.

Both MCP-1 and TGFβ<sub>1</sub> were strongly expressed in MMC and epithelial cells (Figure 6). Expression of MIP-1α was detected in MMC but was not detectable in epithelial cells; similarly, expression of fractalkine and SCF was detected in epithelial cells, but not MMC. Expression of RANTES was detected at very low levels in MMCs and inconsistently in epithelial cells, TECK was detected inconsistently and at low levels in mast cells and epithelial cells.



**Table 2** Primer and probe sequences used for PCR, with references where published sequences are used. Primers for GAPDH were purchased from Stratagene (Amsterdam, The Netherlands). All are known to be intron-spanning with the exception of those marked with an asterisk\*

Target gene	Oligo sequence	PCR product size (bp)	Reference
GAPDH*	Forward 5'-GAAGGGCTCATGACCACAGTCCATG Reverse 5'-TGTTGCTGTAGCCGTATTCATTGTC	454	
MCP-1	Forward 5'-GGAAAAATGGATCCACACCTTGC Reverse 5'-TCTCTTCTCCACCACCATGCAG Probe- 5'-CCAGTGTGACAGTGAAGTGTG	582	(9)
SCF*	Forward 5'-AACTTGGATTATCACTTGCAT Reverse 5'-CAATGCCATGGCTGTCCATTG Probe 5'-TGACCTCGTGTATGC	562†/646‡	(49)
TGFβ <sub>1</sub>	Forward 5'-CGGGGCGACCTGGGCACCATCCATCAG Reverse 5'-CTGCTGCACCTTGGGCTTGCGACCCAC Probe 5'-CTGGAAAGGGCCAGCACCT	406	(23)
RANTES	Forward 5'-TCATCCTCACTGCAGCCGCC Reverse 5'-CTCTATCCTAGCTCATCTCCAAA Probe 5'-CCTCTCCCTCGCGCTGCCTCGTG	254	(9)
MIP-1α	Forward 5'-GAAGAGTCCCTCGATGTGGCTA Reverse 5'-CCCTTTTCTGTTCTGCTGACAAG Probe 5'-CCCCGACTGCCTGCTGCTTCTCC	462	(9)
Fractalkine	Forward 5'-TGGTCCAGAGCTGGCAATAA Reverse 5'-TGGCTTCCTCACTCTCAGGA Probe 5'-GTGTAGGGACTTGTGCATGTGTG	816	(50)
TECK*	Forward 5'-TTTGAAGACTGCTGCCTGG Reverse 5'-GTCTTCTTCTCAACAAGCC Probe 5'-GAGCGCCTCAGACTCTCAGACTG	742	(34)
mMCP-1	Forward 5'-GGAAAAGTGGAGAGAAAGAACCTAC Reverse 5'-GACAGCTGGGGACAGAATGGGG	460	(23)

n, Number of preparations examined. SCF, †secreted form, ‡transmembrane form.

## DISCUSSION

Up-regulation of several chemokines and cytokines has been shown in epithelium in response to viruses (9), bacteria (8,26) and nematode parasites (11,12). In this study, we show constitutive expression of a number of chemokines and cytokines in murine intestinal epithelium, and increased expression of MCP-1 following *N. brasiliensis* infection.

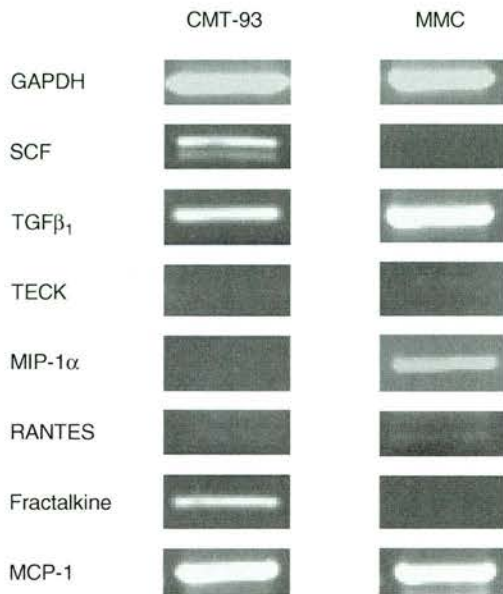
Serum mMCP-1 levels and mMCP-1 mRNA expression in epithelial samples were measured as indicators of mast cell numbers in the jejunum and epithelium. We have previously characterized the mast cell response to *N. brasiliensis* and shown the increase in mast cell numbers to be closely paralleled by serum mMCP-1 levels (19,25), and by jejunal mMCP-1 mRNA expression (25).

The Bjerknes method of epithelial isolation is superior to other chelating, enzymic or mechanical methods because intact pieces of epithelium are recovered with little contamination with lamina propria cells and with high cell viability (21). Our modification of this method was easier to perform and resulted in a similar recovery of epithelium with comparable viability (> 95%). Low contamination of samples with non-epithelial cells was found at all time-points examined.

MCP-1 is well established as a monocyte chemoattractant and has also been shown to be chemotactic for murine IL-3-dependent bone marrow mast cells (7) and mouse skin mast cells (14). Expression of MCP-1 has been correlated with an increase in mast cell numbers in wound healing and in chronic airway inflammation (27,28). In the intestinal tract, MCP-1 expression has been detected by immunohistochemistry in normal colon epithelium (29), lamina propria and endothelial cells (30), making it a potential candidate for mast cell recruitment to the intestine in nematode infection. The high levels of expression of MCP-1 seen in whole gut in this study suggest that it could be involved in initial recruitment of mast cell precursors from the circulation into the tissue, which normally occurs as early as day 3 postinfection (31). Although the increased expression of MCP-1 identified in the epithelium may be important in intraepithelial migration of MMC, it seems unlikely that a significant chemotactic gradient could be established.

The source of expression of MCP-1 and the other molecules examined in the epithelial fraction is unknown, and may include both epithelial cells and intraepithelial inflammatory cells, such as lymphocytes and mast cells. We have





**Figure 6** Expression of MCP-1 and other chemoattractants in cultured mucosal mast cell homologues (MMC) and the CMT-93 mouse intestinal epithelial cell line. Expression of SCF, TGF $\beta_1$ , fractalkine and MCP-1 was detected in CMT-93 cells; whereas expression of TGF $\beta_1$ , MIP-1 $\alpha$  and MCP-1 was detected in MMCs. RANTES was also expressed at very low levels by MMCs and inconsistently by CMT-93 cells. Expression of TECK was detected inconsistently and at very low levels in both cell types.

shown strong expression of MCP-1, TGF $\beta_1$ , SCF, fractalkine and low levels of RANTES and TECK by cultured mouse epithelial (CMT-93) cells, and expression of RANTES (32), TECK (33) and fractalkine (34) has also been demonstrated in various other intestinal epithelial cell types. Expression of MCP-1 (35), MIP-1 $\alpha$  and RANTES (36) has been reported in mast cells, and MMCs cultured in our laboratory also expressed these three chemokines and TGF $\beta_1$ . Expression of these molecules by newly recruited intraepithelial mast cells could contribute to expression in epithelial samples leading to the apparent increase in MCP-1 against the low background expression of this chemokine in epithelium. Intraepithelial lymphocytes (IEL) are able to express MIP-1 $\alpha$ , RANTES and TGF $\beta_1$  (37,38) and may contribute to constitutive expression in epithelial samples. Alterations in IEL numbers following infection were not examined in this study; however, other authors have shown a marked decrease in IEL numbers following infection with the nematode parasite *T. spiralis* (39,40). If a similar decrease was seen following *N. brasiliensis* infection, this would make IELs a less likely source of the chemoattractants examined.

The chemokines MIP-1 $\alpha$  and RANTES, which are also chemotactic for mast cells (4,7), are expressed constitutively

in intestinal epithelium, but up-regulation was not seen following *N. brasiliensis* infection. This was surprising since these molecules have been shown to be up-regulated in other inflammatory diseases, including asthma (41) and inflammatory bowel disease (30,42). Additionally, up-regulation of RANTES expression in a rat model of colitis was correlated with an increase in mast cell numbers, and this increase was abrogated by use of Met-RANTES, a specific antagonist of RANTES receptors CCR1 and CCR5 (43). Constitutive expression of TECK and fractalkine are in agreement with previous studies, which have implicated these molecules in lymphocyte trafficking (33,34), but fractalkine was recently also shown to be chemotactic for mast cells. Expression may therefore contribute towards non-inflammatory recruitment of the small numbers of mast cells that reside in the nonparasitized jejunum. Expression of the cytokines SCF and TGF $\beta_1$  was also measured in epithelial samples since these molecules are both mast cell growth factors (23) and mast cell chemoattractants (17,18). Additionally, SCF expression has been correlated with mast cell accumulation in arthritis (44) and pollen allergies (45), and is up-regulated in intestinal epithelial cell cultures following *Salmonella typhimurium* infection (16). Both molecules were expressed in epithelium, but no alteration in expression was seen following nematode infection.

There was no evidence for a change in expression of the majority of the chemoattractants examined following *N. brasiliensis* infection. One reason for this may be that invasion and damage of epithelial cells is necessary to evoke alteration in inflammatory gene transcription and therefore most studies of the epithelial response to nematodes have focused on invasive parasites. This is also considered to be the case for bacterial infection of the gut, with a lack of inflammatory response to the high bacterial load of normal gut contents (46,47). Alteration of chemokine expression in response to noninvasive parasites may, however, be possible via other mechanisms; excretory-secretory products are key allergens in the immune response to *N. brasiliensis* and those from plerocoids of the parasite *Spirometra erinaceieuropaei* can modulate chemokine expression in macrophages (48). The limitations of RT-PCR in measurement of changes in gene expression must also be considered, with small changes in expression not being detected by the methods used.

The specific recruitment of MMCs following *N. brasiliensis* infection is difficult to explain in the light of this evidence of constitutive expression of several mast cell chemoattractants from intestinal epithelium, especially since preliminary unpublished data from our laboratory suggest that mucosal mast cells express several of the corresponding receptors for the chemoattractants expressed. However, mast cell recruitment may depend on other chemoattractants not investigated, or may be limited by other factors, such as seeding



of mast cell precursors into tissues and expression of the necessary integrins or proteases to allow cells to move through the extracellular matrix.

In conclusion, we have shown both increased expression of MCP-1 following *N. brasiliensis* infection, and constitutive expression of the chemokines MIP-1 $\alpha$ , RANTES, TECK and fractalkine, and cytokines SCF and TGF $\beta$ <sub>1</sub>, in murine intestinal epithelium. Further investigation is required to determine the role of these molecules both in cell trafficking and in the inflammatory response to nematode parasites in the gut.

## ACKNOWLEDGEMENTS

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# TGF- $\beta$ 1 Regulates Adhesion of Mucosal Mast Cell Homologues to Laminin-1 Through Expression of Integrin $\alpha_7$ <sup>1</sup>

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Mucosal mast cells (MMC) or their precursors migrate through the intestinal lamina propria to reside intraepithelially, where expression of mouse mast cell protease-1 indicates the mature phenotype. Alterations in expression of integrins that govern cell adhesion to the extracellular matrix may regulate this process. As the key cytokine mediating differentiation of mouse mast cell protease-1-expressing MMC homologues in vitro, TGF- $\beta$ 1 was considered a likely candidate for regulation of the integrins that facilitate intraepithelial migration of MMC. Therefore, we examined adhesion of bone marrow-derived mast cells cultured with and without TGF- $\beta$ 1 to laminin-1, fibronectin, and vitronectin along with expression of integrins likely to regulate this adhesion. Adhesion of PMA-stimulated cultured mast cells to laminin-1 increased from  $5.3 \pm 3.6\%$  (mean  $\pm$  SEM) in the absence of TGF- $\beta$ 1 to  $58.7 \pm 4.0\%$  ( $p < 0.05$ ) when cultured mast cells had differentiated into MMC homologues in the presence of TGF- $\beta$ 1. Increased adhesion of MMC homologues to laminin-1 was also stimulated by Fc $\epsilon$ RI cross-linking and the calcium ionophore A23187. Expression of the laminin-binding integrin  $\alpha_7$  by MMC homologues grown in the presence of TGF- $\beta$ 1 was demonstrated by RT-PCR and flow cytometry, and preincubation of MMC homologues with the  $\alpha_7$ -neutralizing Ab 6A11 inhibited adhesion to laminin-1 by 98% ( $p < 0.05$ ), demonstrating a novel role for this molecule in adhesion of a hemopoietic cell to laminin-1. *The Journal of Immunology*, 2002, 169: 5689–5695.

Cell trafficking into and through tissues depends upon complex interactions, including adhesion to extracellular matrix (ECM)<sup>4</sup> proteins (1); integrins are the major molecules that mediate this process (2). Mast cells are involved in the pathophysiology of immediate-type hypersensitivity reactions, wound healing, and chronic inflammatory diseases (3, 4). The ability to move through tissues is mandatory for their physiological function, and integrins are likely to be involved in mast cell migration.

The roles of several integrins and other adhesion molecules in mast cell adhesion to ECM proteins has been studied using murine IL-3-dependent, bone marrow-derived cultured mast cells (CMC). These cells adhere to laminin-1 and -2 (5–8), fibronectin (9), and vitronectin (10), and integrins  $\alpha_6\beta_1$  (5, 8),  $\alpha_3\beta_1$  (11), and  $\alpha_v\beta_3$  (10), respectively, have been shown to play a role in this adhesion. Additionally, Abs to the non-integrin 67-kDa laminin binding protein (LBP) block adhesion of IL-3-dependent CMC to laminin-1 (6), indicating a role for this molecule

In vivo, mast cells are phenotypically classified into two major subsets, connective tissue and mucosal (12, 13), and mucosal mast cells are important in the immune response to gut-dwelling nematodes. Nematode infection of the gut results in recruitment of the precursors of mucosal mast cells (MMC) (14) and their migration intraepithelially, where expression of the MMC-specific chymase, mouse mast cell protease-1 (mMCP-1), indicates the mature mucosal phenotype (15). As IL-3-dependent CMC are thought to represent an immature mast cell phenotype (16) and lack mMCP-1 expression (17), they may be a poor model for study of the molecular interactions involved in this process, especially since it is widely recognized that the cytokine stem cell factor (SCF) is a key growth and differentiation factor for mast cells in vivo and in vitro (18). We have recently developed an in vitro model of mast cell differentiation in which addition of the cytokine TGF- $\beta$ 1 to bone marrow cells supplemented with IL-3, IL-9, and SCF regulates differentiation of bone marrow cells into a close homologue of the mucosal phenotype, as shown by abundant expression of mMCP-1 (19).

Adhesion molecule expression in mast cells may be modulated during differentiation. The integrin  $\alpha_4$  is expressed by IL-3-dependent CMC, is down-regulated in older cultures (11, 20), and is not present in mature tissue-derived connective tissue mast cells (20), but is required for the intestinal recruitment of MMC (14). In our in vitro model and in IL-3-dependent CMC (21), TGF- $\beta$ 1 up-regulates the expression of integrin  $\alpha_E$  (22), which may facilitate retention of MMC intraepithelially by binding E-cadherin (23). Therefore, as the key cytokine mediating differentiation of the mucosal phenotype, TGF- $\beta$ 1 may also change the spectrum of integrins expressed to reduce adhesion to ECM proteins and increase adhesion to the basement membrane (BM) protein laminin, thus facilitating intraepithelial migration. In support of this, one study showed that supplementing IL-3-dependent CMC with TGF- $\beta$ 1 for 48 h increased adhesion to laminin-1 (7); the mechanism was thought to be increased adhesion receptor expression.

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<sup>4</sup> Abbreviations used in this paper: ECM, extracellular matrix; BM, basement membrane; CMC, bone marrow-cultured mast cell; LBP, 67-kDa laminin binding protein; MMC, mucosal mast cell; mMCP-1, mouse mast cell protease-1; SCF, stem cell factor.



To test the proposed hypothesis that the adhesion properties of CMC are regulated by TGF- $\beta$ 1, we have compared adhesion of mast cells cultured with and without TGF- $\beta$ 1 to the ECM proteins fibronectin and vitronectin and the BM protein laminin-1. We have also examined the expression of several integrins using RT-PCR and flow cytometry. In addition to those integrins previously implicated in mast cell adhesion, we have investigated the role of the laminin-binding integrin  $\alpha_7$  in adhesion of TGF- $\beta$ 1-dependent CMC. This integrin was originally thought to be skeletal muscle specific (24), but expression of the  $\alpha_{7B}$  isoform has since been found in other tissues, including intestinal epithelium, where it correlates with intestinal cell differentiation (25). In this study we show the expression of  $\alpha_{7B}$  integrin in cultured MMC homologues, which is regulated by TGF- $\beta$ 1. We also demonstrate, by use of the blocking Ab 6A11, a novel role for  $\alpha_7$  in promoting adhesion of mucosal mast cell homologues to laminin-1.

## Materials and Methods

### Mast cell culture

Mouse bone marrow cells were isolated from the femurs of male 12-wk-old BALB/c mice and suspended at  $5 \times 10^5$ /ml in DMEM (Life Technologies, Paisley, U.K.)/10% FCS (Sigma, Poole, U.K.), 2 mmol of L-glutamine, 1 mmol of sodium pyruvate, 100 U/ml of penicillin, 100  $\mu$ g/ml of streptomycin, and 2.5  $\mu$ g/ml of fungizone (Life Technologies). Cells were then supplemented with 5 ng/ml IL-9, 1 ng/ml IL-3 (R&D Systems, Abingdon U.K.), and 50 ng/ml SCF (PeproTech, London U.K.) with or without 1 ng/ml TGF- $\beta$ 1 (Sigma). Mast cells cultured with and without TGF- $\beta$ 1 are termed CMC<sup>T+</sup> and CMC<sup>T-</sup>, respectively. CMC were fed every 2–3 days by centrifuging and resuspending in half-volume of original culture medium and half-volume of fresh medium. Cells used for studies were mature cells, generally 14–30 days old, and consisted of >99% mast cells, as shown by toluidine blue staining. Immunohistochemistry was used to identify mMCP-1-positive cells (15). Typically, <2% of CMC<sup>T-</sup>, but >98% of CMC<sup>T+</sup>, were positive for mMCP-1 expression.

### MC/9 culture

A mast cell line, MC/9, known to express the integrin  $\alpha_6\beta_1$  (5) was cultured for use as a positive control for flow cytometry and for blocking studies using a neutralizing rat anti- $\alpha_6$  mAb (GoH3). MC/9 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM (American Type Culture Collection) with 10% FCS (Sigma) and 10% T-stim (BD Biosciences, Oxford, U.K.), as instructed by American Type Culture Collection.

### Antibodies

Mouse IgG1 mAbs to  $\alpha_7\beta_1$  integrins were obtained by immunization of  $\alpha_7$ -deficient mice with wild-type primary myoblasts, as described previously (26). Clone 6A11 was used for inhibition of CMC<sup>T+</sup> adhesion to laminin, since it has the strongest adhesion inhibition activity of all the anti- $\alpha_7$  clones obtained. Clone 3C12 did not inhibit cell adhesion to laminin and was used for flow cytometry.

Murine IgG1 and rat IgG1 and IgG2a isotype controls, rat anti-mouse  $\alpha_7$  IgG1 (clone RMV-7), rat anti-mouse  $\alpha_5$  IgG2a (clone 5H10-27), rat anti-mouse  $\alpha_6$  IgG2a (clone GoH3), and rat anti-mouse Fc $\gamma$ RIII/II (Fc block, clone 2.4G2) were obtained from BD Pharmingen (Oxford, U.K.). Biotinylated-anti rat IgG2a or IgG1 secondary Abs were also obtained from BD Pharmingen, and anti-mouse IgG1-Alexa Fluor 488 was obtained from Molecular Probes (Leiden, The Netherlands).

### Adhesion assays

Murine Engelbreth-Holm Swarm sarcoma BM laminin-1 and bovine fibronectin and vitronectin were obtained from Sigma, and optimum coating concentrations for these proteins were determined from dose-response studies (data not shown). Ninety-six-well ELISA plates were coated overnight with 100  $\mu$ l of proteins at 20  $\mu$ g/ml in PBS, with the exception of vitronectin, which was used at 10  $\mu$ g/ml; 3% BSA in PBS was used as a control. Excess coating proteins were then removed, and nonspecific binding was blocked by incubation for 2 h at 37°C with 3% BSA in PBS. CMC (and MC/9 cells for blocking studies) were washed twice in PBS/0.1% BSA before resuspending at  $5 \times 10^5$  cells/ml in their respective culture medium (without cytokines). CMC (100  $\mu$ l) were then loaded in quadruplicate into wells. PMA (50 ng/ml; Sigma) was added to some wells im-

mediately after loading to investigate the effect of cell activation on adhesion. Cells were then incubated for 1 h at 37°C in 5% CO<sub>2</sub>, after which nonadherent cells were aspirated, and the wells washed three times with PBS. The number of cells adherent to wells was estimated using the  $\beta$ -hexose aminidase assay (27).

### Regulation of CMC<sup>T+</sup> adhesion to laminin-1

The effect of sensitization with IgE followed by addition of specific Ag and the effect of calcium ionophore A23187 on adhesion of CMC<sup>T+</sup> to laminin-1 were investigated. For stimulation by Fc $\epsilon$ RI cross-linking, CMC<sup>T+</sup> were first sensitized by incubation overnight in complete culture medium with 100 ng/ml of IgE anti-DNP (Sigma). They were then washed twice in PBS/0.1% BSA and resuspended in culture medium (without cytokines) before loading into wells and immediate addition of 10 ng/ml of DNP-human serum albumin (Sigma). Controls included IgE-sensitized cells to which no Ag was added and unsensitized cells to which Ag was added. For stimulation with calcium ionophore, CMC<sup>T+</sup> cells, washed and resuspended in culture medium without cytokines, were loaded into wells, and 1  $\mu$ M calcium ionophore A23187 (Sigma) was immediately added. PMA-stimulated cells were also included as a positive control for stimulation of adhesion, and as a negative control, wells were included where no activating agent was added. The adhesion assay was then performed as described above.

### Analysis of integrin expression by RT-PCR

Expression of integrins by cells from four separate cultures of CMC<sup>T-</sup> and CMC<sup>T+</sup> was investigated by RT-PCR. Total RNA was recovered from  $5 \times 10^6$  cells/culture in Tri-Reagent (Sigma) by phenol-chloroform extraction. RNA was then DNase treated using a DNA-free kit (Ambion, Houston, TX) and 1  $\mu$ g of RNA reverse-transcribed in a 20- $\mu$ l volume using a Promega RT kit (Promega, Southampton, U.K.). One microliter of each RT reaction was used for semiquantitative PCR, employing gene-specific primers (Table I); cycle numbers were optimized so that increases in expression would result in corresponding increases in signal intensity. Negative controls were set up for each sample containing RNA only (no cDNA), and each PCR experiment included a negative control omitting cDNA. PCR products were separated on a 1.4% agarose gel containing 0.5  $\mu$ g/ml of ethidium bromide and were visualized and recorded under UV light using a Kodak Image Station 440cf imaging system (Eastman Kodak, Rochester, NY). PCR product identities were confirmed by Southern hybridization using gene-specific oligonucleotide probes as described previously (28).

### Analysis of integrin expression by flow cytometry

Surface expression of integrins was analyzed by flow cytometry (FACS-Scan; BD Biosciences). To detect the expression of  $\alpha_5$ ,  $\alpha_6$ , and  $\alpha_7$ ,  $1 \times 10^6$  cells were incubated for 5 min with 0.5  $\mu$ g of murine Fc block, followed by incubation for 60 min with 1  $\mu$ g of rat mAb against  $\alpha_5$  (5H10-27),  $\alpha_6$  (GoH3), or  $\alpha_7$  (RMV-7), respectively, or isotype control in PBS/10% mouse serum. The cells were then washed twice in wash buffer (PBS/0.1% BSA) and incubated for 30 min with 1  $\mu$ g of biotin-anti rat IgG2a or IgG1 in PBS/10% mouse serum before washing twice and incubating for 30 min with 100  $\mu$ g of streptavidin-PE (Vector, Peterborough, U.K.). To detect  $\alpha_7$  expression,  $2 \times 10^5$  cells were incubated with 0.1  $\mu$ g of murine Fc block before incubation for 60 min with 2  $\mu$ g of mouse anti- $\alpha_7$  (3C12) or mouse IgG1 isotype control in PBS/5% FCS. They were then washed twice in wash buffer and incubated for 30 min with anti-mouse IgG1 Alexa Fluor 488 diluted 1/2000 in PBS/5% FCS. Following both labeling protocols, cells were washed twice and fixed for 10 min in 2% paraformaldehyde before analysis by flow cytometry; all procedures were performed on ice.

### Blocking studies

The effects of integrin-specific mAbs on CMC<sup>T+</sup> adhesion to laminin-1 were examined. Adhesion assays were as previously described, except CMC<sup>T+</sup> were preincubated with respective mAbs or isotype controls before loading into wells. Preincubation conditions and optimum Ab concentrations were determined in pilot studies (data not shown). To determine the effects of  $\alpha_6$  and  $\alpha_7$  integrins on adhesion of CMC<sup>T+</sup> to laminin-1, CMC<sup>T+</sup> were preincubated for 10 min at room temperature with the  $\alpha_6$  mAb GoH3 (1  $\mu$ g/ml) or for 30 min on ice with the  $\alpha_7$  mAb 6A11 (10  $\mu$ g/ml). MC/9 cells were included as a positive control in experiments investigating the effect of GoH3 on adhesion to laminin-1(5).



Table I. Primer sequences and conditions used for RT-PCR<sup>a</sup>

Gene	Oligo Sequence	Product Size (bp)	Anneal Temperature (°C)	Mg <sup>2+</sup> (mM)	pH	Cycle No.	Ref. No.
$\alpha_3$	Forward: 5'-ATT GAC TCA GAG CTG GTG GAG GAG	$\alpha_{3A}$ 305	63	2.0	8.3	35	50
	Reverse: 5'-TAC TTG GGC ATA ATC CGG TAG TAG Probe: 5'-GCT GGG TTG CTG CTG CTG GG	$\alpha_{3B}$ 161					
$\alpha_5$	Forward: 5'-TTG CTG GAC TGT GGT GAA GAC Reverse: 5'-CTG GTT GAT GAG CTG GTA GAC Probe: 5'-CAG TCT CTG GGG TGG CCT TC	582	55	1.5	8.3	32	51
$\alpha_6$	Forward: 5'-GAG GAA TAT TCC AAA CTG AAC TAC	$\alpha_{6A}$ 399	55	2.5	8.3	35	50
	Reverse: 5'-GGA ATG CTG TCA TCG TAC CTA GAG Probe: 5'-CGA TGC CAC CTA TCA CAA GG	$\alpha_{6B}$ 269					
$\alpha_v$	Forward: 5'-CCG CCG GTG CCA GCC CAT TGA G Reverse: 5'-GCT ACC AGG ACC ACC GAG AAG T Probe: 5'-CTG TGC TCC ATT GTA CCA CT	337	55	1.5	8.3	32	52
$\alpha_7$	Forward: 5'-CAC CGG ATG TCC ATC AGG AC	$\alpha_{7A}$ 480	55	1.5	8.3	35	53
	Reverse: 5'-GTT GCT CAG AGA TGC ATC C Probe: 5'-CTG GTA CTG CTG CTG TGG AA	$\alpha_{7B}$ 366					
$\beta_1$	Forward: 5'-GGC AAC AAT GAA GCT ATC GT	$\beta_{1A}$ 266	55	1.0	8.3	30	50
	Reverse: 5'-CCC TCA TAC TTC GGA TTG AC Probe: 5'-TTG GGA TGA TGT CGG GAC	$\beta_{1D}$ 348					
$\beta_3$	Forward: 5'-CTG GTG TTT ACC ACT GAT GCC AAG Reverse: 5'-TGT TGA GGC AGG TGG CAT TGA AGG Probe: 5'-CCA CAG TGG GAG TCC TGT CT	393	60	2.5	8.9	38	52
LBP	Forward: 5'-CTG AAG TTT GCT GCT GCC ACA Reverse: 5'-GCT GGA TGG GCA CAG AGG GAA Probe: 5'-ATG CCT GAT CTT TAC TTC TA	520	55	1.0	8.3	26	
GAPDH	Forward: 5'-GAAGGGCTCATGACCACAGTCCATG Reverse: 5'-TGTGCTGTAGCCGTATTCATTGTC	454	55	1.5	8.3	20	

<sup>a</sup> Cycle parameters were as follows: denaturation, 30 s at 94°C; annealing, 30 s at temperature indicated in the table; extension, 1 min at 72°C. References are included where published primers are used, and all reactions were performed using a Techne Geneamp thermal cycler (Techne, Cambridge, U.K.).

Statistical analysis

PRISM (version 3.0 for Windows; GraphPad, San Diego, CA) statistical software was used to compare data using the nonparametric Mann-Whitney U test, with a statistical significance level of  $p < 0.05$ .

Results

MMC homologues exposed to TGF- $\beta$ 1 adhere to laminin-1

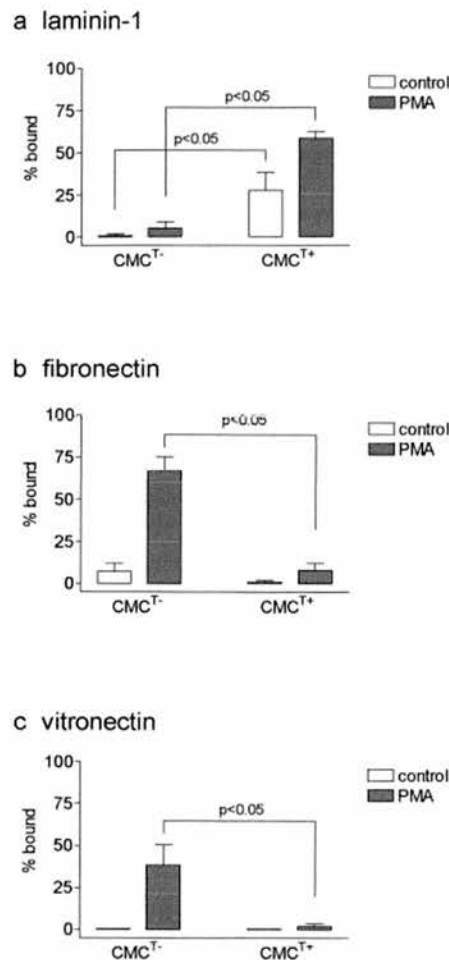
We investigated the effect on their adhesion properties of culturing mast cells with and without TGF- $\beta$ 1 (1 ng/ml). Results are from four separate experiments, each performed in quadruplicate, using CMC from different cultures and are expressed as the mean  $\pm$  SEM of each experiment. The range of means  $\pm$  SEMs of separate experiments is also shown. Experiments established that  $27.7 \pm 10.7\%$  (range,  $5.2 \pm 0.3$  to  $56.4 \pm 2.9\%$ ) of CMC<sup>T+</sup> (mMCP-1<sup>+</sup> MMC homologues) adhered spontaneously to laminin-1. CMC<sup>T+</sup> adhesion to laminin-1 increased to  $58.7 \pm 4.0\%$  (range,  $51.4 \pm 4.6$  to  $69.1 \pm 2.9\%$ ) following PMA stimulation (Fig. 1a), and adhesion of both control (unstimulated) and PMA-stimulated populations of CMC<sup>T+</sup> was significantly greater ( $p < 0.05$ ) than that of equivalently stimulated CMC<sup>T-</sup> (controls,  $0.0 \pm 0.1$  to  $3.5 \pm 2.8\%$ ; PMA-stimulated,  $0.0 \pm 0.8$  to  $15.6 \pm 2.6\%$ ) that were grown in the absence of TGF- $\beta$ 1. In all experiments CMC adhesion to BSA was  $<5\%$  (data not shown). Adherent CMC<sup>T+</sup> flattened and took on a more polarized morphology on laminin-1 (data not shown). PMA-stimulated CMC<sup>T+</sup> adhered poorly to fibronectin ( $7.9 \pm 4.3\%$ ) and did not adhere to vitronectin (Fig. 1b)

Stimulation with Ag/IgE and with the calcium ionophore A23187 up-regulates adhesion of MMC homologues to laminin-1

Adhesion of IL-3-dependent CMC to ECM proteins can be stimulated by treatment with Ag/IgE or calcium ionophore A23187 (7), and similar mechanisms may control adhesion in vivo. We therefore investigated whether the TGF- $\beta$ 1-induced adhesion of CMC<sup>T+</sup> (mMCP-1<sup>+</sup> MMC homologues) to laminin-1 was also regulated by these mechanisms. Both Ag/IgE and ionophore treatments significantly increased ( $p < 0.05$ ) adhesion by CMC<sup>T+</sup> to laminin-1 (Fig. 2). Ag/IgE treatment resulted in flattening and polarization of cells, but after calcium ionophore treatment adherent cells retained a rounded shape (data not shown). This experiment was repeated twice using cells from different cultures, with similar results.

MMC homologues exposed to TGF- $\beta$ 1 express the laminin-binding integrin  $\alpha_{7B}$  at both mRNA and protein levels

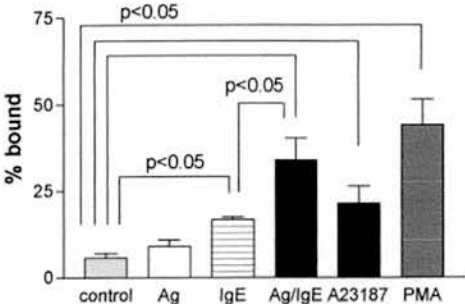
To determine the mechanism of TGF- $\beta$ 1-mediated binding of CMC to laminin, we compared the expression of integrin transcripts by CMC<sup>T-</sup> and CMC<sup>T+</sup> populations. Previous studies (5, 8) have shown involvement of  $\alpha_6$  in adhesion of CMC to laminin; therefore, we examined the expression of this integrin and of the laminin-binding integrins  $\alpha_3$  and  $\alpha_7$  in unstimulated CMC cultured with and without TGF- $\beta$ 1, using semiquantitative RT-PCR. The  $\alpha_7$  primers could amplify both  $\alpha_{7A}$  and  $\alpha_{7B}$  mRNA, resulting in PCR products of 480 and 366 bp, respectively, but only a single band of 366 bp was detected, indicating the expression of  $\alpha_{7B}$



**FIGURE 1.** CMC adhesion to ECM and BM proteins. Adhesion of unstimulated (control) and PMA-stimulated CMC<sup>+</sup> and CMC<sup>-</sup> to laminin-1 (a), fibronectin (b), and vitronectin (c). CMC<sup>+</sup> grown in the presence of TGF- $\beta$ 1 (MMC homologues) adhered to laminin-1, but not to fibronectin or vitronectin, whereas CMC<sup>-</sup> grown in the absence of TGF- $\beta$ 1 adhered poorly to laminin-1, but adhered to fibronectin and vitronectin following PMA stimulation. The adhesion of both control and PMA-stimulated CMC<sup>+</sup> to laminin was significantly greater ( $p < 0.05$ ) than the adhesion of equivalently stimulated CMC<sup>-</sup>. Also, the adhesion of CMC<sup>-</sup> to fibronectin and vitronectin was significantly greater ( $p < 0.05$ ) than the adhesion of PMA-stimulated CMC<sup>+</sup>. Results are the mean  $\pm$  SEM of four separate experiments, each performed in quadruplicate and using cells from four different cultures.

transcripts only. The expression of  $\alpha_7$  integrin was low to undetectable after 35 cycles of PCR in CMC<sup>-</sup>, whereas inclusion of TGF- $\beta$ 1 in cultures resulted in a substantial up-regulation of  $\alpha_7$  mRNA expression by CMC<sup>+</sup> (Fig. 3). The expression of  $\alpha_7$  integrin was confirmed by flow cytometry using the mouse mAb 3C12. Surface expression of  $\alpha_7$  was consistently detected in TGF- $\beta$ 1-supplemented CMC<sup>+</sup> from three different cultures during separate experiments, whereas no expression was detected in CMC<sup>-</sup> (Fig. 4).

Transcripts for other laminin-binding integrin subunits, 3A and 6A, and of the very late Ag integrin  $\beta$ -subunit  $\beta_1$  were expressed at similar levels in both CMC<sup>+</sup> and CMC<sup>-</sup> (Fig. 3). However, because others have reported the expression of  $\alpha_6$  integrin in murine CMC and the mast cell line MC/9 (5, 8, 10), we compared  $\alpha_6$  expression in MC/9 cells and CMC with or without TGF- $\beta$ 1 by flow cytometry. The expression of  $\alpha_6$  was virtually absent in CMC<sup>+</sup> and CMC<sup>-</sup>, but, as expected,  $\alpha_6$  expression was high in

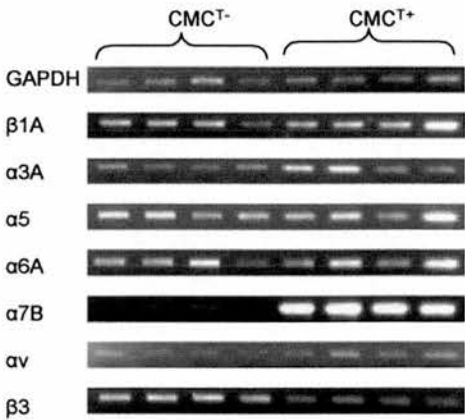


**FIGURE 2.** Regulation of adhesion of MMC homologues to laminin-1. Adhesion was significantly increased ( $p < 0.05$ ) by stimulation using PMA, Ag/IgE, and the calcium ionophore A23187. Overnight sensitization of MMC homologues with IgE also significantly increased adhesion to laminin, but addition of specific Ag to sensitized cells significantly increased adhesion above that seen in cells sensitized only. Results are the mean  $\pm$  SEM ( $n = 4$ ) of one experiment that was repeated twice more using cells from different cultures, with similar results.

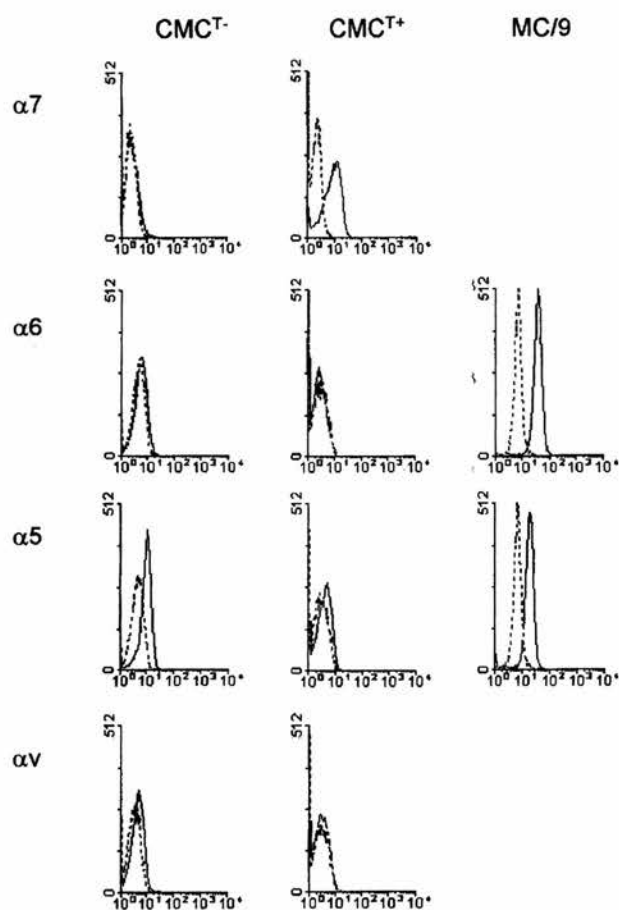
MC/9 cells (Fig. 4). LBP transcripts were also detected at similar levels in both cell types (data not shown).

*The  $\alpha_7$ -neutralizing mAb 6A11 blocks adhesion of MMC homologues to laminin-1*

To demonstrate the role of integrins in adhesion of MMC homologues to laminin, CMC<sup>+</sup> were preincubated with neutralizing Abs before use in adhesion assays. The  $\alpha_7$ -neutralizing mAb 6A11 at 10  $\mu$ g/ml resulted in a 98% reduction in adhesion of PMA-stimulated CMC<sup>+</sup> (MMC homologues) to laminin-1 (Fig. 5a), which was statistically significant ( $p < 0.05$ ). This experiment was repeated twice more in quadruplicate and triplicate, with reductions in adhesion of 98 and 100%, thus clearly indicating a role for  $\alpha_7$  in adhesion to laminin-1. 6A11 also blocked spontaneous adhesion to laminin-1 (not shown) and adhesion following Ag/IgE and A23187 stimulation (Fig. 5, b and c). The use of neutralizing Abs has shown a role for  $\alpha_6$  in adhesion of IL-3-dependent CMC to laminin-1 (5, 8, 10), but the anti- $\alpha_6$  mAb GoH3 (1  $\mu$ g/ml) had no effect on adhesion of CMC<sup>+</sup> to laminin-1 (Fig. 5d), whereas adhesion of MC/9 cells, which are reported to express the integrin  $\alpha_6\beta_1$  (5) and were used as a positive control for this Ab, was reduced by 98% in the presence of GoH3.



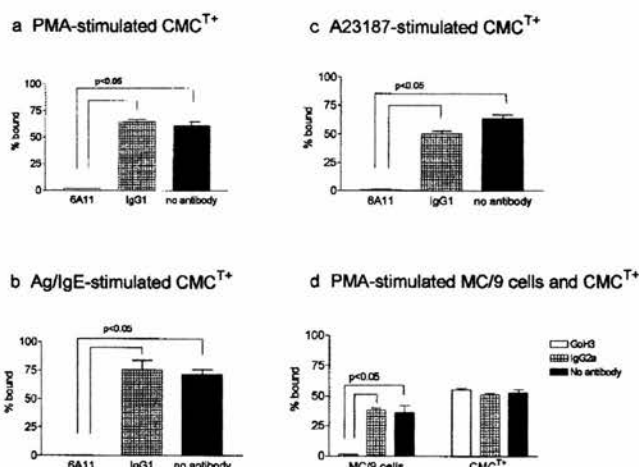
**FIGURE 3.** Analysis of adhesion molecule expression in CMC<sup>-</sup> and CMC<sup>+</sup> by semiquantitative RT-PCR. Expression of  $\alpha_7$  integrin mRNA was highly up-regulated in CMC cultured with TGF- $\beta$ 1, whereas TGF- $\beta$ 1 moderately down regulated the expression of integrin  $\beta_3$ . Transcripts for other adhesion molecules were expressed at comparable levels by both CMC<sup>-</sup> and CMC<sup>+</sup>.



**FIGURE 4.** Analysis of integrin expression in  $\text{CMC}^{\text{T}-}$  and  $\text{CMC}^{\text{T}+}$  by flow cytometry. MC/9 cells were included as positive controls for the expression of  $\alpha_5$  and  $\alpha_6$  integrins.  $\text{CMC}^{\text{T}+}$  expressed  $\alpha_7$  integrin, whereas there was no expression in  $\text{CMC}^{\text{T}-}$  cultured without TGF- $\beta$ 1.  $\alpha_6$  expression was virtually absent in both  $\text{CMC}^{\text{T}-}$  and  $\text{CMC}^{\text{T}+}$ ; high expression was detected in the positive control MC/9 cells.  $\alpha_5$  integrin was expressed by  $\text{CMC}^{\text{T}-}$ , but there was decreased expression in  $\text{CMC}^{\text{T}+}$  and high expression in MC/9 cells. The expression of integrin  $\alpha_v$  was virtually absent in both  $\text{CMC}^{\text{T}-}$  and  $\text{CMC}^{\text{T}+}$ . These experiments were repeated twice more using cells from separate cultures, with similar results.

#### *Mast cells cultured in the absence of TGF- $\beta$ 1 adhere to fibronectin and vitronectin, but not to laminin-1*

Previous studies of the binding of murine CMC to matrix proteins have predominantly used cells cultured in the presence of IL-3 alone (5, 7, 8, 10, 11). Adhesion of CMC grown in a combination of IL-3, SCF, and IL-9, all of which are expressed in the gut (29, 30) and are known mast cell growth factors (31–33), has not previously been studied. As shown above, CMC grown in the absence of TGF- $\beta$ 1 did not adhere to laminin-1. Furthermore, these cells did not adhere spontaneously to vitronectin, but had low ( $7.1 \pm 4.9\%$ ; range,  $0.5 \pm 1.0$  to  $21.8 \pm 4.3\%$ ) spontaneous adhesion to fibronectin (Fig. 1*b*). However, after PMA stimulation,  $\text{CMC}^{\text{T}-}$  adhered to vitronectin ( $38.4 \pm 12.2\%$ ; range,  $8.3 \pm 3.6$  to  $65.6 \pm 6.0\%$ ) and fibronectin ( $66.8 \pm 8.6\%$ ; range,  $47.9 \pm 6.3$  to  $82.6 \pm 1.4\%$ ) at levels which were significantly greater ( $p < 0.05$ ) than those observed for  $\text{CMC}^{\text{T}+}$  (Fig. 1, *b* and *c*). Adhesion of  $\text{CMC}^{\text{T}-}$  to both ECM proteins was associated with cell flattening (data not shown).



**FIGURE 5.** Role of integrins in the adhesion of  $\text{CMC}^{\text{T}+}$  (MMC homologues) to laminin-1 (20  $\mu\text{g}/\text{ml}$ ). The  $\alpha_7$  integrin-neutralizing Ab 6A11 (10  $\mu\text{g}/\text{ml}$ ) reduced the adhesion of PMA-stimulated  $\text{CMC}^{\text{T}+}$  to laminin-1 by 98% ( $p < 0.05$ ; *a*) and similarly reduced the adhesion of  $\text{CMC}^{\text{T}+}$  stimulated by Ag/IgE (*b*) and that of the calcium ionophore A23187 (*c*) to laminin-1. In contrast, the  $\alpha_6$ -neutralizing Ab GoH3 (1  $\mu\text{g}/\text{ml}$ ) had no effect on the adhesion of  $\text{CMC}^{\text{T}+}$  to laminin-1, but significantly ( $p < 0.05$ ) reduced the adhesion of MC/9 cells to laminin-1 by 98% (*d*). Results are the mean  $\pm$  SEM ( $n = 4$ ) for single experiments. Experiments *a* and *d* were repeated twice more using cells from different cultures, with similar results.

#### *Mast cells cultured in the absence of TGF- $\beta$ 1 do not express $\alpha_7$ integrin, but show increased expression of fibronectin- and vitronectin-binding integrins*

As previously described, analysis of integrin expression by RT-PCR established that  $\text{CMC}^{\text{T}-}$  do not express  $\alpha_7$  integrin, but express  $\beta_{1A}$ ,  $\alpha_{3A}$  and  $\alpha_{6A}$  at comparable levels to those seen in  $\text{CMC}^{\text{T}+}$  (Fig. 3). However, as  $\text{CMC}^{\text{T}-}$  adhered to fibronectin and vitronectin, we wondered whether this was also due to TGF- $\beta$ 1-mediated alterations in integrin expression. We therefore investigated the expression of integrins  $\alpha_5$  and  $\alpha_v\beta_3$  in  $\text{CMC}^{\text{T}-}$  and  $\text{CMC}^{\text{T}+}$  by RT-PCR, as these integrins play a role in the adhesion of IL-3-dependent CMC to fibronectin and vitronectin, respectively. The expression of  $\alpha_5$  and  $\alpha_v$  transcripts was similar in both cell types, but the expression of the vitronectin-binding integrin  $\beta_3$  was up-regulated in  $\text{CMC}^{\text{T}-}$  (Fig. 3) compared with that in  $\text{CMC}^{\text{T}+}$ . In the absence of differences at the mRNA level, flow cytometry was used to investigate surface expression of  $\alpha_5$  and  $\alpha_v$  (Fig. 4). Expression was measured on cells from three different cultures of  $\text{CMC}^{\text{T}-}$  and  $\text{CMC}^{\text{T}+}$  during separate experiments, and the mast cell line MC/9, which has been shown previously to express integrin  $\alpha_5$  (11), was used as a positive control for the expression of this integrin. Surface expression of  $\alpha_5$  was increased in  $\text{CMC}^{\text{T}-}$  compared with  $\text{CMC}^{\text{T}+}$ , but the expression in both CMC was lower than seen in the positive control MC/9 cells. The expression of  $\alpha_v$  was low to absent in both  $\text{CMC}^{\text{T}-}$  and  $\text{CMC}^{\text{T}+}$ .

## Discussion

CMC have previously been shown to adhere to laminin (6, 7), fibronectin (9), and vitronectin (10). Here we show TGF- $\beta$ 1 modulation of adhesion of CMC to these proteins and a novel role for  $\alpha_7$  integrin in adhesion of MMC homologues to laminin-1. We propose that TGF- $\beta$ 1-mediated up-regulation of  $\alpha_7$  expression in conjunction with differentiation of the mucosal mast cell phenotype and expression of mMCP-1 and  $\alpha_E\beta_7$  (22) may have a role to play in the intraepithelial location of MMC *in vivo*.



$\alpha_7\beta_1$  has been described as being essentially muscle specific (34), and although several non-muscle locations have been described (35, 36), expression has not previously been shown in leukocytes of any subset. Additionally, TGF- $\beta$ 1-mediated regulation of  $\alpha_7$  expression has not been shown in any cell type, and although developmental regulation of  $\alpha_7$  has been described in muscle and has been proposed for intestinal epithelium (25), the molecular signals controlling expression in these cells are unknown.

The  $\alpha_{7B}$  isoform expressed by MMC homologues is also expressed in skeletal myoblasts; the  $\alpha_{7A}$  isoform is restricted exclusively to mature skeletal muscle (26, 37, 38). However, as both isoforms promote adhesion on laminin-1 and laminin-2/4 (39, 40), which may be rich in epithelial BM (41),  $\alpha_{7B}$  expression in vivo could either promote intraepithelial migration or limit egress of MMC from the epithelium into lamina propria via adhesion to BM laminin. Additionally, restricted expression of  $\alpha_{7B}$  by epithelial cells of the crypt-villous junction (25), the primary location of intraepithelial MMC in vivo (42), suggests that  $\alpha_7$  may be important for the adhesion of both epithelial cells and mast cells to the BM at this site.

The phorbol ester PMA stimulated maximal adhesion of MMC homologues to laminin-1, presumably due to alterations in receptor affinity or cytoskeletal rearrangements (43) and the anti- $\alpha_7$  mAb 6A11 blocked this adhesion. Fc $\epsilon$ RI cross-linking and use of the calcium ionophore A23187 also stimulated adhesion to laminin-1, as previously shown in IL-3-dependent CMC (7, 8), and these may represent in vivo mechanisms by which adhesion could be stimulated. Spontaneous adhesion of CMC<sup>T+</sup> to laminin-1 was observed in some cultures, although this was inconsistent and may have been due to low levels of endotoxin contamination, as LPS has been shown to cause mast cell activation (44). Preincubation with 6A11, however, almost completely blocked adhesion via all the above pathways, showing a universal role for integrin  $\alpha_7$  in the adhesion of MMC homologues to laminin-1.

Previous studies (5, 8) have implicated integrin  $\alpha_6$  in the promotion of murine mast cell adhesion to laminin-1 and -2; however, these studies used several mast cell lines and IL-3-dependent CMC, which may be more representative of immature mast cells. The mMCP-1<sup>+</sup> CMC<sup>T+</sup> closely resemble intraepithelial MMC (15, 19), and while these cells expressed  $\alpha_6$  transcripts at similar levels to CMC<sup>T-</sup>, flow cytometry showed low  $\alpha_6$  expression in both cell types. This made  $\alpha_6$  an unlikely candidate for regulation of CMC<sup>T+</sup> adhesion to laminin, and inclusion of the anti- $\alpha_6$  mAb GoH3 in adhesion assays had no effect on adhesion of CMC<sup>T+</sup> to laminin-1. Comparison with positive  $\alpha_6$  expression by flow cytometry and GoH3-mediated inhibition of adhesion to laminin-1 in MC/9 cells further supports our findings that  $\alpha_6$  integrin plays no role in the adhesion of MMC homologues to laminin-1.

Human skin mast cells also adhere to laminin, but do not significantly express  $\alpha_6$  integrin, and adhesion is inhibited by Abs to  $\alpha_3$  integrin (45). Abs to  $\alpha_3$  integrin were not included in our studies, but in view of conclusive evidence for the role of  $\alpha_7$  integrin in the adhesion of MMC homologues and because the expression of  $\alpha_3$  mRNA expression in CMC<sup>T+</sup> was similar to that in CMC<sup>T-</sup>, which do not adhere to laminin, a major role for  $\alpha_3$  integrin in laminin binding seems unlikely in MMC. The importance of different integrins in adhesion to laminin may vary between mast cell phenotypes, with  $\alpha_6$  possibly playing a role only in the adhesion of immature mast cells, while  $\alpha_3$  and  $\alpha_7$  may mediate the adhesion of mature connective tissue mast cells and MMC, respectively, to laminin.

LBP has been implicated in the adhesion of IL-3-dependent CMC to laminin-1 (6, 7), but the expression of LBP mRNA was similar in both CMC<sup>T-</sup> and CMC<sup>T+</sup> (data not shown). However,

it is reported that LBP expression is post transcriptionally regulated (16); therefore, a cofactor role in the adhesion of MMC homologues to laminin-1 is possible, as suggested in other cell types (47).

CMC cultured in the absence of TGF- $\beta$ 1 (CMC<sup>T-</sup>) do not have a recognizable in vivo counterpart since they lack mMCP-1 and do not show any morphological resemblance to the very well characterized serosal mast cell population. Their role in this study was simply as a comparator for MMC homologues cultured in TGF- $\beta$ 1, and they bound preferentially to fibronectin and vitronectin, but not to laminin. Post-transcriptional down-regulation of the fibronectin-binding integrin  $\alpha_5$  and transcriptional down-regulation of the vitronectin-binding integrin  $\beta_3$  in MMC homologues compared with CMC<sup>T-</sup> suggest that TGF- $\beta$ 1 may also regulate mast cell adhesion to these ECM proteins by alteration in expression of specific integrins.

In vivo, secreted TGF- $\beta$ 1 must be activated to form a functional molecule, and epithelially expressed integrin  $\alpha_v\beta_6$  has been implicated in this process (48). Our most recent studies have shown coexpression of TGF- $\beta$ 1 and integrin  $\alpha_v\beta_6$  in murine jejunal epithelium, and that  $\beta_6^{-/-}$  mice have significantly reduced numbers of intraepithelial MMC following *Nippostrongylus brasiliensis* infection (49). This result is highly suggestive of a role for TGF- $\beta$ 1 in the intraepithelial MMC response to nematode parasites. It is possible that mechanisms include regulation of the expression of integrins, including  $\alpha_7\beta_1$  and  $\alpha_E\beta_7$ , that could be critical for intraepithelial migration and retention of MMC.

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